THE BIOCHEMICAL CHARACTERISATION OF EXPRESSED BOVINE MHC CLASS I MOLECULES.

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DECLARATION:

I hereby declare that the work presented in this thesis is the products of my own efforts, and has not been submitted in any previous application for a degree. The work on which it is based is my own except where stated in the text and the acknowledgments section.

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ABSTRACT

The bovine MHC (BoLA) class I region has been studied mostly by serology. The products defined by the allo-antisera were thought to be alleles of a single highly polymorphic locus. However, studies involving the use of molecular biological and biochemical techniques revealed the presence of a second expressed locus. Furthermore, the use of one dimensional isoelectric focussing reveals complicated patterns of molecules from each animal which indicates that the bovine class I encoding region is much more complex than previously thought. The aim of this project was to investigate the origins of the charge diversity of BoLA class I molecules observed using 1D-IEF.

The BoLA class I molecules appear to be glycosylated at a single N-linked position and that the carbohydrate moiety attached is of the complex type with up to three terminal sialic acid residues. The results also indicated that phosphorylation does not occur in class I molecules immunoprecipitated from resting bovine PBL. Furthermore, neither modification mentioned above is responsible for the observed charge heterogeneity. Additionally, 2D analysis of the immunoprecipitated samples suggested that alternative splicing is unlikely to be the major contributor to the observed complexity.

In order to investigate whether the different charge variants had different primary sequences, peptide mapping was employed. Different BoLA charge variants had distinct peptide maps. Animals sharing part of their serotype also show similarities in the digestion patterns they exhibited. These experiments suggested that

each serological specificity is composed of a number of different polypeptides that represent the products of more than one class I locus. These results and further work involving the visual comparison of the 1D-IEF types of different animals obtained from immunoprecipitations with the MAb W6/32 and the different bovine allo-antisera suggested the existence of strong linkage disequilibrium among the class I molecules expressed by different loci in our herd. The number of different charge variants with different peptide maps indicated that the BoLA system has at least three expressed class I loci.

Additional studies involving the computer analysis of the available bovine class I primary sequences revealed that the secondary structures of these sequences are similar to those of the molecules of the HLA system. Furthermore, the number of residues in the transmembrane region (TM) of class I molecules in locus specific, the inspection of the BoLA sequences revealed them to cluster in three distinct groups with 37, 36 and 35 residues in their TMs respectively. This analysis gives support to the evidence produced in this thesis that at least three BoLA class I loci are present. However, these programmes with the available information did not allow us to identify locus specific structural motifs.

The significance and general implications of these findings are discussed.

CONTENTS:

LIST OF ABBREVIATIONS:

AAS	*Allo-Antisera*
APS	*Ammonium Presulphate*
BoLA	*Bovine Lymphocyte Antigens*
DH_2O	*Distilled Water*
EDTA	*Ethylenediaminetetraacetic acid*
FACS	*Fluorescent Activated Cell Sorting*
g	*Gravity*
HBSS	*Hank's Balanced Salt Solution*
hrs.	*Hours*
IEF	*Isoelectric Focussing*
L	*Litre*
MEM	*Minimum Essential Medium*
min.	*Minute*
ml	*Millilitre*
mM	*Millimolar*
1D	*One Dimensional*
PBS	*Phosphate Buffered Saline*
PAGE	*Polyacrylamide Gel Electrophoresis*
PMSF	*Phenylmethyl-Sulfonyl Fluoride*
SDS	*Sodium Dodecyl Sulphate*
sec.	*Second*
2D	*Two Dimensional*
U	*Unit*
ul	*Microlitre*

CHAPTER ONE
Literature review

1. LITERATURE REVIEW:

1.1. INTRODUCTION:

The major function of the vertebrate immune system is to limit or eliminate infectious agents. One of the fundamental aspects of the immune system is its specificity, in that it can react to individual molecules and/or agents. Three genetically related families of antigen-binding molecules have evolved which impart this specificity, Immunoglobulins (Ig), the T cell receptor (TcR) and the major histocompatibility molecules (MHC). All are surface bound glycoproteins associated with the cells of the immune system. Ig and TcR are antigen-specific receptors for B cells and T cells respectively and the binding of specific antigen to these molecules results in the clonal expansion of these cells. The MHC molecules are essential accessory molecules in the interaction of antigen with the TcR.

Within the MHC family there are two major subfamilies of molecules which are biologically functional under normal physiological conditions. MHC class I and MHC class II are genetically linked but structurally and functionally diverse. Although their function is the binding of antigen and its presentation to T cells, the two types of MHC molecules bind antigen in different cellular compartments and present those peptides to distinct T cell populations.

One feature of MHC molecules that distinguishes them from any other family of molecules is the considerable degree of polymorphism maintained in both classes of molecules. Polymorphism in the MHC has a profound effect on the extent and quality of the immune response. Most allelic variation in both types of molecules is

found to be concentrated in the membrane distal portions, α_1 and α_2 for class I molecules and α_1 and β_1 for class II molecules. This site is functionally important for antigen presentation, or restriction of the T cell response. More polymorphism in that site means a better capacity to respond to a wider variety of pathogens (for reviews see Kappes and Strominger,1988 and Lawler et al.,1990).

1.2. THE BIOLOGICAL FUNCTION OF MHC MOLECULES:

The first function that the MHC molecules are involved in before disease combat is the shaping of the T cell repertoire which determines the subsequent ability to discriminate between self and non-self. In the thymus, T cells will die a programmed death if their receptor fails to bind any ligand. The T cells are deleted if their receptor binds to MHC plus specific peptide. The cells could, on the other hand, be rescued from programmed death or deletion if their receptor binds to MHC ligands in the thymic epithelium with an unknown peptide content. As a consequence of the latter event, the cells will develop into either $CD4^+$ or $CD8^+$ depending on the specificity of their TcR. The mutually exclusive expression of CD4 or CD8 genes by the mature cells is regulated by unknown molecular events (Pardoll and Carrera,1992).

MHC molecules have a number of other important biological functions. The first observation which drew attention to this fact was graft rejection. When a donor expresses a determinant different from the recipient, the latter's immune system recognises the mismatch and the graft is rejected. Both class I and class II MHC molecules have a central part to play in this mechanism (for review see Mason and

Morris,1986). Direct evidence for the function of MHC molecules as restriction elements came from the observation that when mice of various H-2 types were injected intracerebrally with Lymphocytic Choriomeningitis Virus (LCMV), all showed severe neurological disease seven days later. When the cytotoxic activity of their spleen cells was tested on virus infected L-cells (a fibroblast cell line of C3H ($H\text{-}2K^k$) origin) it was observed that only spleen cells carrying the same H-2 specificity had the ability to lyse the target L-cells specifically (Zinkernagel and Doherty,1974a,b; Doherty and Zinkernagel,1974,1975). The combination of the concepts of MHC restriction and antigen presentation (Shimonkeritz et al.,1983; Townsend et al.,1986), led to the view that the MHC molecules bind processed antigen fragments, forming ligands for TcR. In 1985 Babbitt and co-workers gave life to the above model by demonstrating that direct binding does occur between specific antigen peptides from hen egg lysozyme and the murine class II molecule $I\text{-}A^k$. A similar relationship was demonstrated between the immunogenic peptide derived from chicken ovalbumin and the $I\text{-}A^d$ molecule. Matzinger (1981) and Schwartz (1985) developed a model to explain MHC restriction. In this model the TcR was postulated to interact with the complex of a peptide bound to a particular MHC molecule, whether class I or class II, leading to the activation of the effector cell. The T cell selected by one combination of peptide and MHC would only be restimulated by the same or a closely related peptide/MHC complex. The specificity of restriction would then result from variability in the MHC molecule (polymorphism), which determines both the peptide capable of binding and the TcR selected.

1.3. THE STRUCTURE AND ASSEMBLY OF MHC MOLECULES:

1.3.1. The Class I Molecules:

The three dimensional structure of three human MHC class I and one murine class I MHC molecules (HLA-A2, HLA-Aw68, HLA-B27 and H-2K^b), have been elucidated by X-ray crystallography. The structure of the murine molecule has a high degree of similarity to those of the HLA molecules, although individual domains can have slightly altered dispositions (Bjorkman et. al.,1987a; Garrett et al.,1989; Madden et al.,1991; Fremont et al.,1992; Zhang et al.,1992). A description of one of these molecules (HLA-A2) will be given here. The mature MHC molecule (M_r 44,000) has three external domains (α_1-α_3), a transmembrane domain and a cytoplasmic tail. The external domains associate with the non-MHC encoded β_2-microglobulin (β_2m) (fig.1).

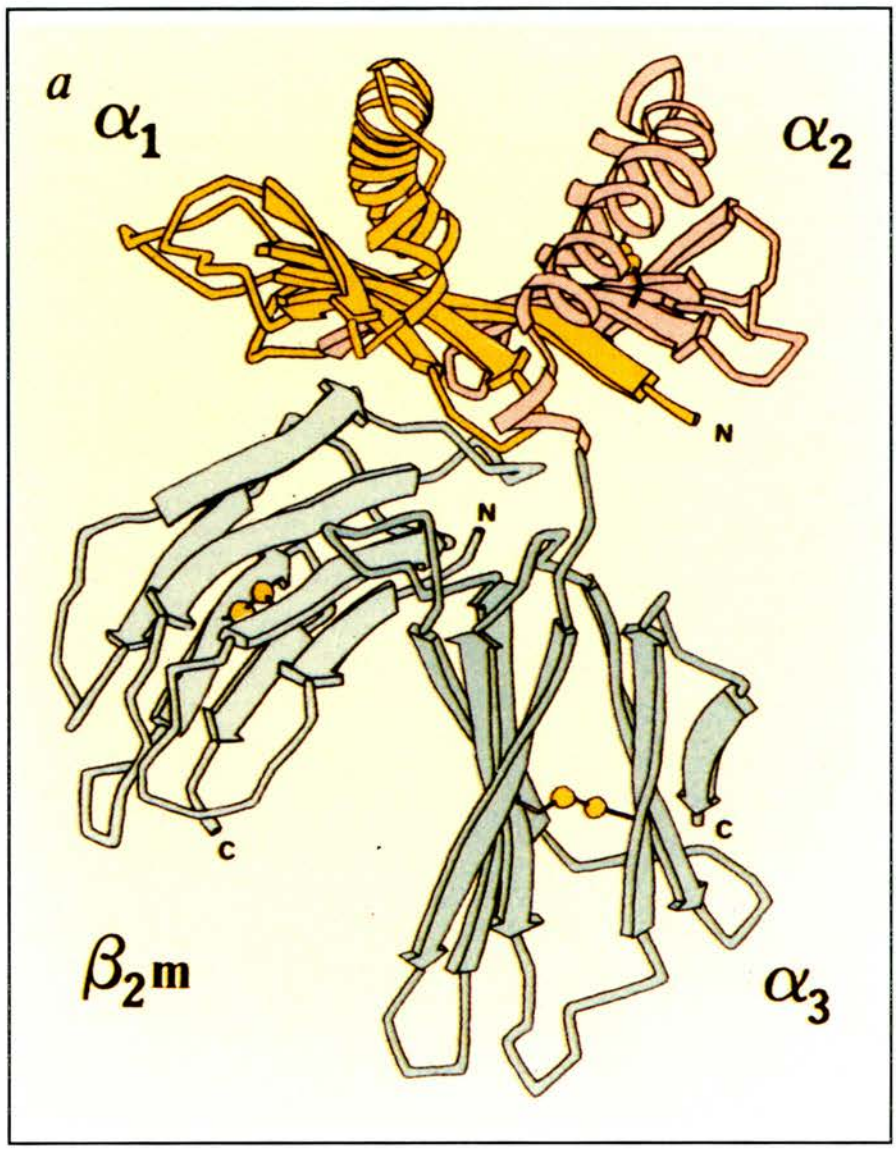
The α_3 and β_2m are proximal to the membrane and are folded into β-sandwich structures (resembling Ig constant domains), each composed of two antiparallel β-pleated sheets connected by an internal disulphide bond. One sheet has four β-strands, while the other sheet has three. The α_3 domain is related to β_2m by a 146° rotation followed by a 1.3nm translation along the rotation axis direction instead of the expected 180° rotation without the translation that relates pairs of antibody domains as shown in figure 2 (Bjorkman et al.,1987a). β_2m has more extensive contacts with the α_1-α_2 platform than does the α_3 domain. This is due to the effect of the 1.3nm translation that relates the two Ig-like domains which results in the lifting of β_2m to support the platform.

FIGURE 1.1
Facing page

A SCHEMATIC REPRESENTATION OF THE STRUCTURE OF THE PAPAIN CLEAVED HLA-A2 MOLECULE.

The molecule is shown with membrane proximal membrane domains, α_3 and β_2m, at the bottom, and the polymorphic domains α_1 and α_2 at the top. The C-terminus of the α_3 domain indicated in the figure is the papain cleavage site. The membrane-bound molecule extends past the cleavage point towards the membrane by another 13 amino acids. The polymorphic domains form a platform with a single eight stranded β-pleated sheet (seen edge on), covered by α-helices.

The β-strands are shown as thick arrows in the amino and carboxyl direction, α-helices are represented as helical ribbons. Connecting loops are depicted as thin lines. Disulphide bonds are indicated as two connected spheres.



To summarise, α_3 only interacts with β_2m which in turn interacts with both membrane distal domains forming the platform.

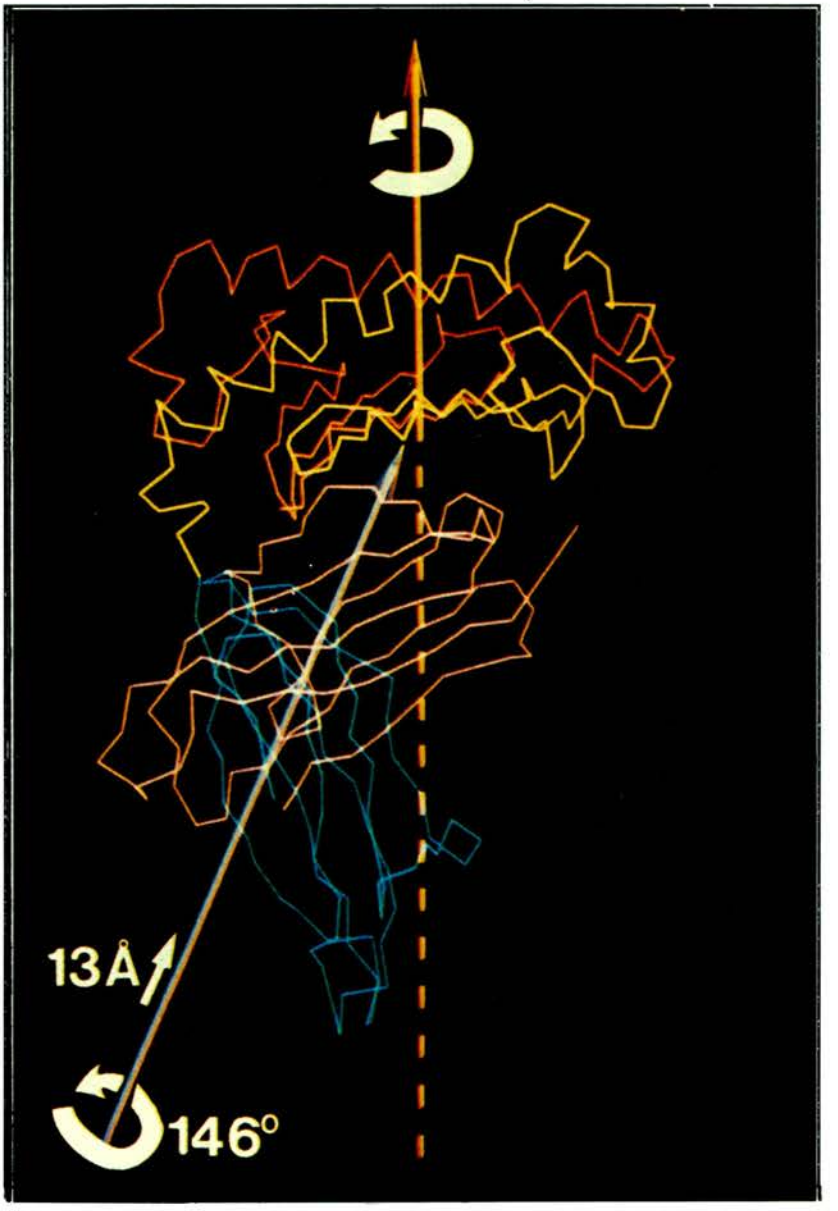
The α_3 domain, plays an important role in the selection of antigen-specific cells. This role arises from the unique juxtaposition of the Ig-like domains, which are thought to be an adaptation for binding CD8, which directly interacts with α_3 (Garret et al.,1989). An exposed loop involving residues 223-229 appears to be a major contact site of the α_3 domain with the CD8 α chain, with residue 227 having the most effect on the binding (Salter et al.,1990; Potter et al.,1989 and Connolly et al.,1988). Position 233 is also thought to have a direct effect on the association of α_3 with CD8, other residues, such as 235,245 and 247 are thought to have an indirect effect on the association (Salter et al.,1990). CD8, if inhibited by monoclonal antibodies, interferes with the process of T-cell selection which supports the findings of Garret and co-workers (1989) that the α_3 domain plays a role in the selection process (Aldrich et al.,1991).

The distal part of the MHC class I molecule is comprised of the α_1 and α_2 domains of the heavy chain which share a common tertiary structure. Each is composed of a four-stranded antiparallel β-pleated sheet forming the amino-terminal half of the domain, followed by a long α-helical region. When α_1 and α_2 are paired in the HLA structure, they are related by a nearly exact two-fold rotation axis (177° rotation followed by 0.07nm translation), so that a single eight-stranded antiparallel β-pleated sheet is formed from the four-stranded sheets in each domain. The α-helices then lie on top of the sheet, antiparallel to each other, their helical axis making an angle of approximately 40° with respect to the β-strands in the sheet (fig.2).

FIGURE 1.2
Facing page

LOCATION OF THE PSEUDO-SYMMETRY AXES IN THE HLA-A2 MOLECULE.

The polymorphic domains α_1 (red) and α_2 (yellow) are related by an approximate dyad axes of symmetry (orange arrow) so that a single eight-stranded antiparallel β*-pleated sheet (the floor of the antigen binding site) is formed. The relationship is a rotation of approximately 177° followed by a 0.07nm translation. Domains α_3 (blue) and β_2m (purple) are related by 146° rotation (blue arrow) followed by a 1.3nm translation which results in the lifting of β_2m to support the platform formed by the polymorphic domains. The two axes do not intersect, and deviate from colinearity by 25° (in projection).*



The helical regions of both the α_1 and α_2 domains are composed of two α-helices. The first helix in α_1 is a short helix of 1.5 turns that meets a longer helix of 7.5-8 turns at an angle of approximately 110°. The α_2 domain's helical region also starts with a short helix (3 turns) that meets a longer helix of about 7.5 turns at an approximate angle of 130°. The short helices at the amino-termini of the helical regions in α_1 and α_2 form the means by which the β-pleated sheet (floor of the domain) is linked to the top of the domain (the long helices). The long helices of these two domains are of 4.1nm and 3.7nm respectively, and are located 1.8nm apart. The mean interaxial distance for two helices placed next to each other is (approximately) 1nm (the peptide binding groove). The axes of the two long helices make an angle of approximately 20 with respect to each other, so that the amino-terminal residues of these helices project to the highest point of the molecule, and may therefore be the accessible residues to the TcR recognising the top surface of the peptide/MHC complex (Bjorkman et al.,1987a). The peptide binding groove is a gap around 2.5nm deep. The sides of the gap are formed from the side chains of the two long α-helices of the α_1 and α_2. The bottom is formed from side chains of the central β-strands of the α_1-α_2 β-sheet. The groove is located on the top surface of the molecule, it is lined with both polar and non-polar side chains several of which could be critical for T cell recognition. Figure 3 shows a peptide in the groove (Bjorkman et al.,1987b). The assembly of class I molecules occurs in the endoplasmic reticulum (ER). Both class I subunits (heavy chain and β2m) are guided to the ER by classical amino-terminal signal sequences which are cleaved off once the molecules have been co-translationally translocated across the ER membrane (Pleogh et al.,1979).

FIGURE 1.3
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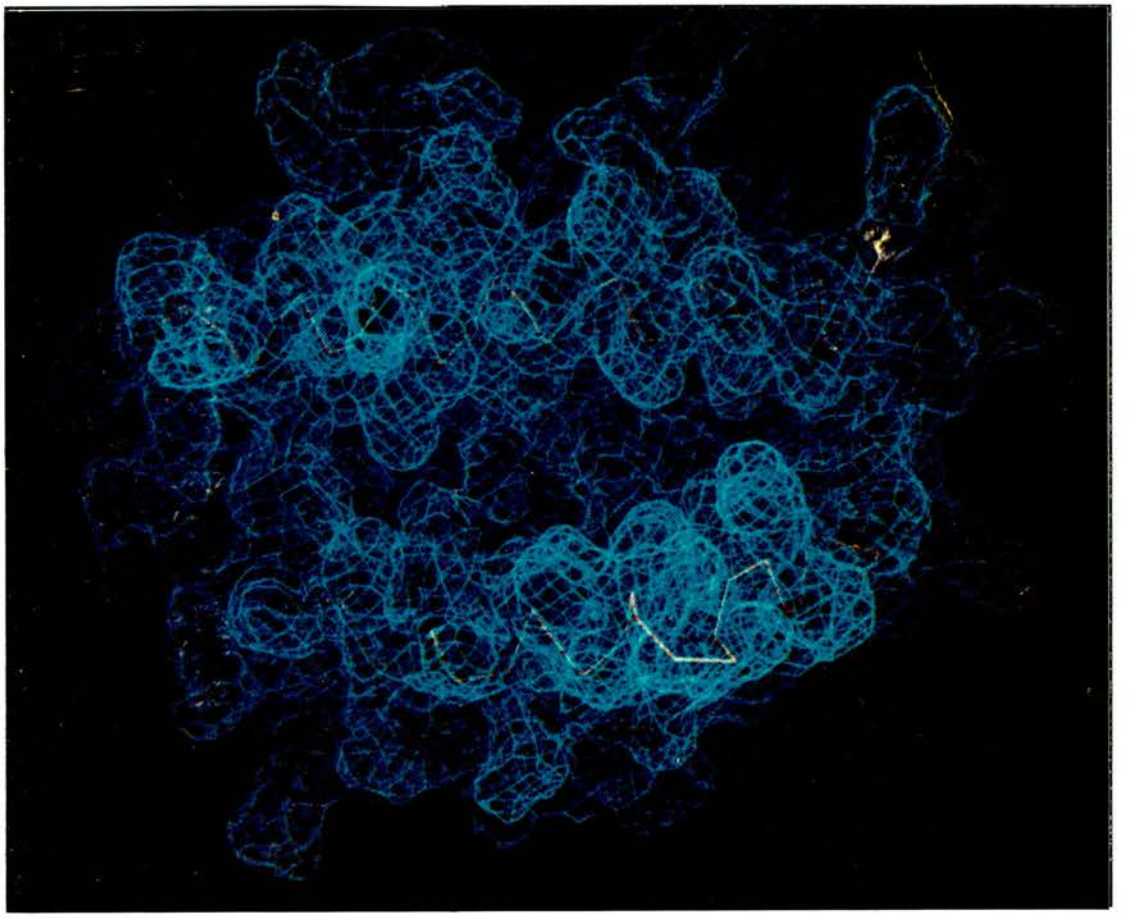
VAN DER WAALS SURFACE REPRESENTATION OF THE ANTIGEN RECOGNITION SITE AT THE TOP OF THE HLA-A2 MOLECULE.

The peptide binding site is a gap around 2.5nm deep and approximately 1.8nm wide. The sides of the gap are formed by the side chains of the two long α*-helices of* α_1 *and* α_2 *domains. The bottom is formed from side chains of the central* β*-strands of the* α_1-α_2 β*-sheet.*



Experiments with cell lines lacking β_2m (Pleogh et al.,1979; Koller et al.,1990; Zijlstra et al.,1990), indicated that these cells lack class I expression as well as $CD8^+$ T cells. Class I heavy chains that dissociate from β_2m at the cell surface readily acquire β_2m from the surrounding medium pointing to the stabilising role played by β_2m (Rock et al.,1991; Vitiello et al.,1990 and Hansen et al.,1988). Some exceptions are H-$2D^b$ and H-$2L^d$ molecules which do not require β_2m for their transport and subsequent surface expression, although its presence may still influence the conformation and increase the stability of expression of these molecules (Krangel et al.,1979; Allen et al.,1986; Myers et al.,1989 and Williams et al.,1989). Until recently, however, it has been generally assumed that MHC class I heavy chains cannot be transported to the cell surface in a form that can be recognised as antigen-presenting molecules in the absence of β_2m. Glas and co-workers (1992) reported that cells deficient in β_2m can present major and minor histocompatibility antigen epitopes for $CD8^+$ T cells and their precursors. The interpretation of these results was that a fraction of MHC class I chains can be transported to the cell surface without β_2m, either alone or in association with short peptides. These heavy chains fall below the threshold for a clear signal in antibody binding assays and usually also below the threshold for recognition by $CD8^+$ T cells induced by MHC class I-positive stimulator cells. However, they are sufficient to trigger certain T-cell clones in the afferent phase of the response, at least in vivo. These clones expand and are able to recognise heavy chains (with or without antigenic peptide) in β_2m^- cells also in the effector phase. This interpretation is in line with the notion that as few as 200 MHC-antigen ligands per cell are enough for detection by T cell (Rosemary-Christinck et al.,1991). Townsend and co-workers (1989) used a

mutant cell line (RMA-S), which only expresses 1/20 of the number of class I molecules on normal cells, to study the effect of antigenic peptides on the assembly of these molecules. They reported that exposing these mutants to certain peptides partially restores the association of the heavy chains (D^b in this case) with β_2m and their expression on the cell surface. Further work, using an in vitro system suggested that the addition of peptides to detergent extracts from these mutant cells enhances the assembly of class I molecules. The peptides are thought to induce conformational changes in the α_1 and α_2 domains of the heavy chain and their association with β_2m. This change is influenced by the concentrations of both peptide and β_2m (Townsend et al.,1990). It was found that specific short peptides, 9-10 amino acids (a.a.) in length, can induce folding of the heavy chain in the absence of β_2m. Both these short and longer peptides (15a.a) can stabilise preformed low-affinity heterodimers (Silver et al.,1991; Ortz-Navarrete and Hemmerling,1991). Fahnestock and co-workers (1992), suggested that secreted empty class I molecules are both immunologically reactive and structurally similar to peptide-carrying class I molecules. Additionally, it was reported that a significant portion of empty class I molecules do exist at the cell surface at physiological temperatures. However, class I molecules with peptides appeared to be more stable than the empty molecules (Fahnestock et al.,1992). The class I molecules are transferred to the trans-Golgi reticulum (TGR) where they are glycosylated before their final journey to the cell surface (Parham et al.,1975). The time before the class I molecules appear on the cell surface varies from 10-60mins depending on the expressing locus, with HLA-B the fastest and HLA-C the slowest, these differences follow the speed of subunit assembly of the different class I molecules (Neefjes and

Ploeph,1988). Using cell lines that have defects in their assembly and transport of class I molecules, it was reported that in these cells the class I molecules are recycled between the ER and Golgi, with a steady-state distribution favouring the ER. Morphological and subcellular fractionation evidence suggested that the class I molecules pass through an organelle or compartment called the "cis-Golgi network" that sorts the protein and selects those for ER retention (Hsu et al.,1991). Degan and Williams (1991) reported that virtually all newly synthesised class I heavy chains associate with an 88,000 protein (p88) to form a complex which is a precursor of the mature class I molecule. This complex exists transiently ($t_{1/2}$=20-45 mins.), and the dissociation of p88 occurs at a distinct rate for each class I molecule examined. This slow dissociation occurs mostly after association with β_2m. The kinetics of p88 dissociation from the class I heterodimers correlates with the characteristic ER to Golgi transport rate for the H-2 molecules examined, suggesting that this dissociation step is the rate limiting step in the ER to Golgi transport. Although this step appears to be a prerequisite for export of these molecules from the ER, other rate limiting steps cannot be totally excluded.

1.3.2. The Class II Molecules:

The structure of this class of molecules is far from fully understood. Unlike class I molecules, no crystal structure for class II has been produced. The structural analysis, therefore, has depended on the available sequences for class II molecules. These molecules exist as heterodimers of non-covalently associated chains, α (M_r

33,000) and β (M_r 29,000). They have a domain structure similar to class I, but the four domains of the class II chains are arranged on two polypeptide chains of roughly the same size that span the membrane bilayer. The membrane-proximal domains of each chain are immunoglobulin-like, with homology to α_3 and β_2m of class I molecules. The N-terminal domains of the class II chains presumably contain the binding site for antigenic peptides, and one of these domains (β_1) has weak sequence homology to class I α_1 and α_2 (Bjorkman et al.,1987a). Because of the homology in sequence and antigen-presenting function between class II and class I, it is proposed that their respective three dimensional structures are similar. By imagining an attachment of the class I α_1 domain to β_2m, a four domain model for class II can be produced. Such class II molecules would have a cleft between the α helices of its α_1 and polymorphic β_1 domains with the bottom of the cleft formed by the N-terminal β-strands of each domain (Brown et al.,1988). Further studies on the function of mouse class II molecules (I-A) agree with the proposed model above (Brown et al.,1990). Class II molecules preferentially bind peptides of 13-18 a.a.. These peptides mostly derive from cellular proteins (self) or from proteins in the external environment. It is suggested that if the class II molecules were to have a similar groove to that of class I molecules, it must be open from one or either side to be able to accommodate the lengths of peptides proposed. One end of the groove in class I molecules is occluded by an invariant tyrosine, the other has an invariant salt bridge. The class II molecules may have a salt bridge at one end of the groove, but the other end has neither a potential salt bridge nor an invariant tyrosine or other bulky side chain which could explain the longer peptides binding to them (Rudensky et al.,1991;

Hunt et al.,1992b).

Like the class I molecules the class II molecules are assembled in the ER and only properly assembled αβ heterodimers leave the ER (Kvist et al.,1982; Sant et al.,1991). During biosynthesis a third chain, called the invariant or gamma chain, associates transiently with the αβ heterodimer and is responsible for the targeting (signal found in cytoplasmic tail) of the heterodimers to the endocytic processing route. It is also responsible for preventing the association of class II with immunogenic peptides in the ER. This association is not essential for the assembly and surface expression of the heterodimer but it makes the process more efficient (Bakke and Doberstein,1990, Lotteau et al.,1990; Teyton and Peterson,1992; Miller and Germain,1986; Roche and Cresswell,1990). It is argued that antigen processing mutant cell lines such as T2 lack the characteristic stability that class II molecules usually show in SDS solutions. The likely reason is thought to be the inability of these molecules to associate with antigenic peptide. The isolation of class II molecules from the T2 cell line revealed that a large proportion of them are associated with invariant chain derived peptides, the longest of which is 23 amino acids long (positions 81-104). Truncated peptides (by about three amino acids) from the same region were also found to be associated with the HLA-DR3 molecule. HLA-DR3 molecules from this cell line can be efficiently loaded with antigenic peptides by exposure to a low pH. However, the addition of the defined class II-associated invariant chain peptide (CLIP) inhibits this association. Therefore, it was suggested that the CLIP may actually define the region of the invariant chain responsible for obstructing the class II binding site (Riberdy et al.,1992). These findings were confirmed with work on the cell line

721.174 (Sette et al.,1992).

Roche and co-workers (1991) reported that the class II molecules are transported from the ER in a complex consisting of three pairs of trimers ($\alpha\beta$ hetrodimer plus invariant chain). During the transfer from the TGR to the endocytic route the invariant chain is degraded by endosomal proteases (Blum and Cresswell,1988; Pieters et al.,1991). The rate of breakdown of the invariant chain determines the speed of transfer of the hetrodimer through the endocytic route and takes 1-3 hours (Neefjes et al.,1990).

1.4. PEPTIDE PROCESSING AND PRESENTATION:

Although the response of $CD8^+$ T cells to a variety of virus infections could be shown to differ with various viruses, the exact nature of the molecules recognised by the $CD8^+$ T cells was until recently, unclear. It was thought that the $CD8^+$ T cells recognised foreign glycoprotein inserted alongside MHC molecules in the membrane of the target cell (Gething et al.,1978; Koszinowski et al.,1977; Wabake-Bunoti & Fan,1983). However, recent advances have revealed this notion to be untrue, showing that class I-restricted T cells recognise degraded fragments of foreign proteins which have passed through the cytoplasm of the target cell (Townsend et al.,1985,1986; Maryanski et al.,1986; Townsend et al.,1986). Degradation of foreign molecules into fragments also occurs in the context of class II MHC molecules, however, this pathway is pH dependent and therefore different from the processing pathway of class I molecules (Unanue,1984; Grey and Chesnut,1985; Beven,1987 and Unanue and Allen,1987).

The need for both class I and II molecules to present degraded protein is consistent with the fact that both $CD8^+$ T cells and $CD4^+$ T cells rely on the same gene pool for the V genes of their TcRs (Davis and Bjorkman,1988).

1.4.1. Processing of Peptides to be complexed with Class I:

Recent evidence pointing to the importance of the cytosol as the location for the processing machinery of the class I molecules came from Moore and co-workers (1988). In their experiments, C57BL/6 ($H\text{-}2^b$) mice immunised against a syngeneic tumour cell and transfected with chicken ovalbumin (OVA) cDNA gave rise to $H\text{-}2K^b$ restricted $CD8^+$ T cells specific for the OVA peptide 258-276. This synthetic peptide and CNBr fragments of OVA were able to target $H\text{-}2^b$ cells for lysis by $CD8^+$ T cells. On the other hand cells incubated with native OVA for up to 24hrs did not become sensitised for recognition and lysis. However, the direct introduction of OVA into the cytoplasm of the cells by osmotic lysis of the pinosomes brought the K^b determinant back into action. Similar evidence implicating the cytoplasm as a "theatre of operations" was reported by Yewdell and co-workers (1988) with work involving directing $CD8^+$ T cell activity against a non-infectious influenza virus. Furthermore, the transfection of minigenes expressing only the relevant epitope resulted in recognition of the pre-processed antigenic site by class I but not class II MHC restricted T cells. This indicates that the native antigen is not required for transport of the epitope into the exocytic pathway (Sweetser et al.,1989). Furthermore, Townsend and co-workers (1986) working on the same virus reported that cells transfected with an influenza haemagglutonin gene lacking its signal peptide are still

lysed by $CD8^+$ T cells. In these cells haemagglutinin is produced and degraded in the cytoplasm. More importantly, the core glycosylation of the protein was also lacking in the transfected cells indicating that only degraded forms of the protein enter the ER.

The use of Brefaldin A, blocks protein transport from the ER by destroying the Golgi complex and completely abolishes the ability of target cells to present the required epitopes for $CD8^+$ T cells lysis. This suggests that the ER is the location where the processed protein encounters class I molecules. These findings all raise the question "how do the antigenic fragments get into the ER?" (Nutchtern et al.,1989).

Studies involving the use of the mutant cell line RAM-S (has 1/20 of the normal levels of class I expression) showed that a pre-Golgi compartment is responsible for the loading of antigenic peptide onto class I molecules. This coupling induces a conformational change which partially restores the expression of class I molecules. This points to the importance of the tripartite structure (heavy chain/ β2m/ peptide) for the expression of stable class I molecules at physiological temperatures (Townsend et al.,1989). The ability of these cells to restore class I expression suggests that they are defective in their ability to provide sufficient peptide for the association with class I molecules, either due to a defect in the processing pathway or in the transport of these peptides into the secretory pathway (Cerundolo et al.,1990; Townsend et al.,1990; Elliot et al.,1991). The study of the class II region of the MHC in mice, rats and humans revealed the presence of two genes with limited polymorphism that encode proteins which belong to the family of transport proteins (Deverson et al.,1990; Trowsdale et al.,1990; Spies et al.,1990; Colonna et al.,1992). The nomenclature of these genes and their products has now been unified across

species as shown in table 1 (The WHO Nomenclature Committee,1992).

The transfection of mutant cell lines RAM-S and 721.134, with cDNA encoding TAP1 and TAP2 restores the ability of these cells to express class I molecules (Spies and DeMars,1991; Attaya et al.,1992). It is thought that TAP1 and TAP2 form a complex spanning the ER membrane and that both of them are needed for peptide transport. Kleijmeer and co-workers (1992) reported that the transporter complex is indeed located in the endoplasmic reticulum and cis-Golgi with the orientation of the ATP-binding domain in the cytosol. These findings are consistent with the proposed transporter role. The function of peptide transport was thought to be ATP dependent (Kelly et al.,1992; Spies et al.,1992). However, Levy and co-workers (1991) reported that while ATP is essential for the loading of peptide onto class I molecules it is not needed for the translocation of peptide across the ER membrane. In addition to the transporter genes, the class II region of the MHC is also found to harbour two genes that encode two proteins forming part of the Low Molecular Mass Polypeptide (LMP) complex (Monaco and McDivitt,1982, Monaco and McDivitt,1984). This complex is thought to be involved in the proteolytic breakdown of foreign peptides into class I binding fragments. This complex is closely related but not identical to the proteasome, an intracellular complex which has multiple proteolytic activities (Brown et al.,1991; Marteinez and Monaco,1991). The proteasome and LMP appear to share a large number of common subunits. This may explain the ability of anti-proteasome antibody to immunoprecipitate both molecules.

TABLE 1.1: New nomenclature of MHC encoded transporter genes.

SPECIES	TAP1	TAP2
HUMAN	Y3, PSF1, RING4	Y4, PSF2, RING11
MICE	Ham1	Ham2
RATS	mtp1	mtp2

* TAP= Transporter Associated with antigen Processing.

The possibility of direct interaction between the LMP complex and the TAP transporter is given credibility by the fact that the two polymorphic TAP genes and the two polymorphic genes encoding subunits of the LMP (LMP-2 and LMP-7) are closely linked within the class II region of the MHC system (Marteinez and Monaco,1991; Glynn et al.,1991; Kelly et al.,1991 and Cho et al.,1991). However, Arnold and co-workers (1992) and Momburg and co-workers (1992) using the lymphoblastoid cell lines 721.174 and T2 respectively (both of which have a deletion in the class II region that encompasses both the TAP and LMP haplotypes) reported that the transfection of the TAP genes into these cells is enough to restore the stable expression of MHC class I molecules and suggested that the LMP genes were not generally required in that pathway. However, the fact that LMP-2 and LMP-7 are both subunits of a larger proteolytic, complex and that they and other subunits of the proteasome/LMP complex are regulated by IFN-γ, indicates that other molecules in the complex could also have a role in the production of antigenic peptides (Yang et al.,1992). Further, it could be argued that the absence of only two subunits (LMP-2 and LMP-7) might not affect the ability of the complex to generate antigenic peptides. There is limited information on the proteolytic specificity of the LMP/Proteasome complex. Dick and co-workers (1991) showed that the complex can produce amongst others nonameric peptides which are tailored for the binding of class I molecules (Brown et al.,1991).

More recently Henderson and co-workers (1992) extracted peptides bound to HLA-A2.1 molecules on the antigen processing mutant cells CEMx721.174.T2 (T2). The analysis of these peptides revealed only seven dominant sequences as opposed to

over 200 associated with normal cells expressing HLA-A2.1. These peptides which were derived from signal peptide domains of normal cellular proteins, were usually larger than 9 residues, and were also associated with normal cells expressing HLA-A2.1. These findings suggested that the proteolysis of signal peptide domains in the ER could be a second mechanism for processing and presentation of peptides for association with class I molecules. These results were independently confirmed by Wei and Cresswell (1992). An overall picture of the class I proteasome-dependent processing pathway is given in figure 4.

The production of truncated proteins as a by-product of protein or RNA synthesis is another mechanism for generating endogenous peptides for presentation with class I molecules. This is consistent with the observation that promoterless genes encoding tumour specific antigens sensitize target cells for lysis by tumour specific $CD8^+$ T cells following the transfection of the genes into the cell (DePlaen et al.,1988; Sibille et al.,1990 and Szikora et al.,1990). In 1989 it was suggested by Boon and Van Pel that another mechanism exists for the generation of endogenous antigenic peptides. These peptides could be generated directly by the translation of very short genetic regions. It was proposed that short genetic regions (peptons), located around the sequence coding for the peptide, can be transcribed autonomously. The peptons would have the length of a few hundred base pairs, thereby exceeding only by a small factor that of the sequence coding for the peptide. Peptons would be transcribed by a polymerase that does not require the promoter sequences initiating eukaryotic genes. The resulting pepton-RNA would then be translated to produce a peptide that would either bind directly to class I molecules or do so after the precise cleavage of some

segments. As a consequence, antigenic peptides could be produced in the absence of classical transcription of the corresponding gene and therefore in the absence of the normal protein product of the gene (Boon and Van Pel,1989). If peptons are a major route of entry of determinants into the class I processing pathway, then some determinants should be derived from out-of-frame sequences. No sequences of that nature have been found. This evidence suggested that peptons either do not exist, or are never created at levels sufficient to trigger a response. Such levels are only reached from transfected DNA; they represent prematurely terminated translation products (Yewdell and Bennink,1992).

1.4.2. The processing of peptides for the class II Pathway:

Class II MHC molecules differ in both function and distribution from class I. It follows, therefore, that their pathways of antigen processing are also different.

It is generally accepted that the class II molecules derive their peptides predominantly from exogenous sources, by endocytosis, and that the subsequent degradation process is acid-dependent and occurs in the endosome or lysosome. Zeigler and Unanue (1982) showed that neutralisation of the acidic compartments of the processing cell using agents such as chloroquine, primaquine and NH_4Cl inhibits antigen presentation by class II but not class I MHC molecules. However, these drugs also have an effect on the proper processing and transport of the MHC molecules themselves and also on the morphology of the endosomes and the trans-Golgi reticulum (Peters et al.,1991; Pieters et al.,1991; Nowell and Quaranta,1985; Neefjes and Ploegh,1992; Wood et al.,1991; Lippencott-Schwatz et al.,1991).

FIGURE 1.4
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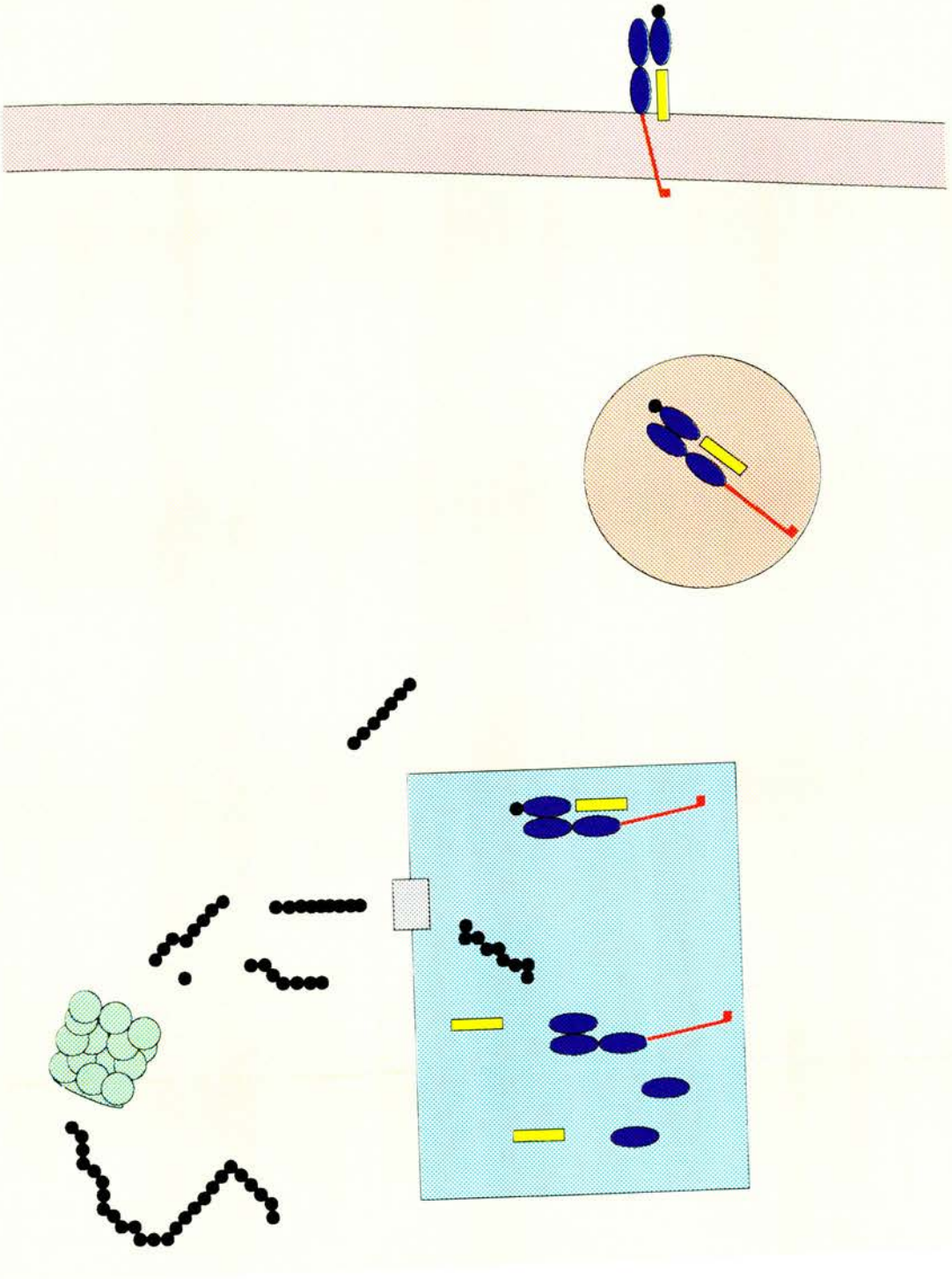
AN OVERVIEW OF THE CLASS I PROCESSING PATHWAY.

Endogenous proteins (black) are processed in the cytoplasm via a proteolytic complex (proteasome) (green) into smaller peptides. The peptides are transported across the ER membrane through transporter molecules (grey). In the ER (sky blue) the peptides associate with the class I heavy chains (navy blue and red) and β_2m (yellow). The complex of peptide/heavy chain/β_2m is then transported through golgi vesicles (orange) to the cell surface (pink).



To avoid such side effects Neefjes and Ploegh (1992) used the combination of a neuraminidase protection assay, designed to measure surface appearance of molecules in the endocytic route (Neefjes et al.,1990), with the exposure of the isolated class II molecules to SDS at room temperature. The results suggested that stable class II αβ dimers are formed in an endocytic compartment while en route to the plasma membrane, but prior to appearance at the cell surface. The stabilisation of αβ dimers in SDS could result from the binding of peptide to the class II molecules (Sadegh-Nasserei and Germain,1991; Stern and Wiley,1992). Only the newly synthesized pool of class II molecules, not the pre-existing cell surface pool, associates with peptides derived from internalised antigen. This excludes a major functional role for the fraction of recycled class II molecules in presentation of unprocessed exogenous antigen (Davidson et al.,1991).

The study of the localisation of this binding step has been fraught with difficulties, due to the imprecise definition of the endocytic route's subdivisions. Peters and co-workers (1991) showed an abundance of class II molecules in the late endocytic route implicating either the endosomes or lysosomes (both part of the endocytic route) as possible sites for the binding of class II and antigen. Furthermore, work by the same group involving the use of double and triple labelling with early and late endocytotic markers revealed that a lysosomal subcompartment is the most likely location for such an interaction with large concentrations of class II molecules present. Other evidence from work involving the use of human and mouse APCs further supported the involvement of a late endocytic compartment in functional antigen presentation by class II molecules (Davidson et al.,1991; Roosnek et al.,1988

and Harding et al.,1991).

These findings contrasted those of Guagliardi and co-workers (1990) who suggested that it is an early endosomal compartment not a late one that is responsible for the binding process.

The involvement of class II molecules in the presentation of endogenous peptides has also been reported where an antigen is modified such that it is retained in the ER (Weiss and Bogen,1991; Brooks et al.,1991). Some of these antigens may subsequently be released from the ER and degraded in the endocytic route or, as was reported by Armstrong (1991) and Dice (1990), the peptides or proteins could be delivered directly to the lysosome via the cytosol.

Recently, Malanti and co-workers (1992) defined two distinct pathways for the presentation of endogenous peptides by class II molecules. The first is similar to the class I presentation pathway using short cytosolic peptides in a TAP-dependent fashion. This process however, is different from the class I pathway in its sensitivity to chloroquine. In addition, its extent and efficiency is not yet known. The second pathway is TAP-independent and may involve the delivery of larger cytosolic molecules to an endosomal/lysosomal compartment for processing, as was suggested previously by Jaraquamada and co-workers (1990) and Long (1992).

A process termed autophagy could also be responsible for the delivery of endogenous peptides to class II molecules. This process permits the uptake of cytosolic material, followed by a fusion between the autophagocytic structure and lysosomal elements. The cytosolic proteins are then degraded and coupled to the class II molecules for presentation. This pathway represents a flexibility in the class II

presentation process that allows it to treat both exogenous and endogenous molecules on equal footing (Gordon and Seglen,1988; Dunn,1990a,b).

1.4.3. Presentation of foreign peptide:

i: Class I presentation:

It was noted when comparing the x-ray crystallographic structures of HLA-A2 and HLA-Aw68 that most of the differences were concentrated in the antigen binding cleft (Garret et al.,1989). The determination of the structure of HLA-B27 and that of H-2K^b revealed that the cleft is occupied by a nonamer peptide (an octamer, as well as, a nonamer was found in the cleft of the H-2K^b molecule) in an extended conformation as seen in fig.5 (Madden et al.,1991, Fremont et al.,1992). The extended conformation of bound peptides allows extensive hydrogen bonding with the peptide main chain. Helical conformations, on the other hand, cannot establish the same number of favourable intermolecular interactions because of their intramolecular backbone hydrogen bonds (Rognan et al.,1992). Reddehase and co-workers (1989), using a technique of systematic delimitation of an antigenic site, identified a pentapeptide motif as the minimal antigenic determinant presented by class I molecules and recognised by the TcR. However, nonamer peptides form more stable and active complexes with class I molecules and it is generally accepted that peptides 8-10 residues long preferentially bind to class I molecules (Tsomides et al.,1991; Schumacher et al.,1991; Elliot et al.,1991). More recently, however, Guo and

FIGURE 1.5
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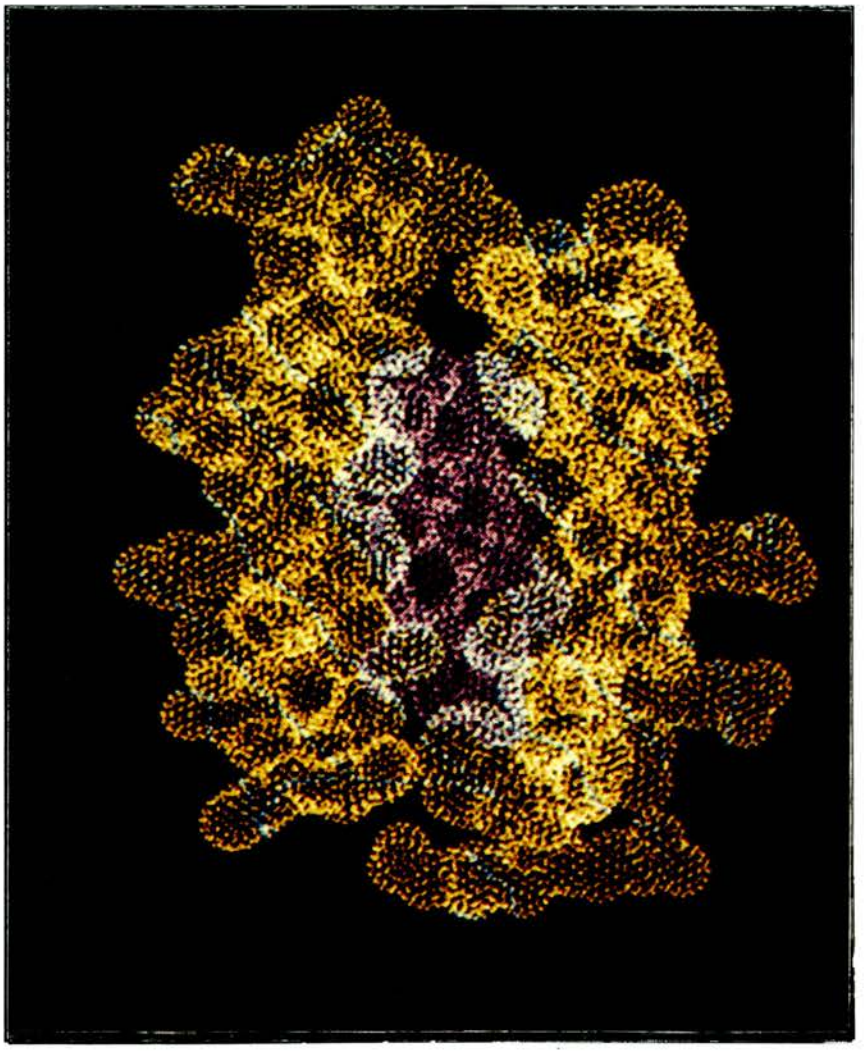
PEPTIDE BOUND TO A CLASS I MHC MOLECULE.

The carbon-α backbone of the α_1 and α_2 domains is shown in the blue with Van der Waals highlighting of the two α-helices (yellow). Van der Waals surface of a hypothetical bound peptide is shown between the two helices (pink). The peptides usually bind class I molecules in an extended conformation.



co-workers (1992) reported that peptides of up to 11 residues can bind HLA-Aw68. X-ray crystallography at resolution 0.19nm showed the atomic structure at the first three and the last two a.a. with no connecting electron density. This suggested that the bound peptides were held at both ends (P2 and the carboxyl terminal residue) and compensate for their extra length by bulging out in the middle (Guo et al.,1992). Silver and co-workers (1992) reported that peptide Np 91-99 of the influenza virus nucleoprotein binds to HLA-Aw68 with the residues at either end (P1, P2, P3 and P9) of the peptide substantially buried in the peptide binding site. The residues in the middle of the peptide, P4-P7, make no hydrogen bonds to the HLA molecule and P4 and P5 do not even make van der Waals contacts. Eleven polymorphic MHC residues contact P1 and P2 and eight other polymorphic MHC residues contact P9, the rest of the peptide contacts only ten MHC residues. In the area of the peptide where contact to HLA is sparse, water molecules provide a hydrogen-bonding bridge to HLA (Silver et al.,1992). Apart from the residues at either end of the peptide, all the other residues are available for TcR recognition. This general mode of interaction is also seen with HLA-B27 (Madden et al.,1991; Jardetsky et al,.1991 and Madden et al.,1992), HLA-Aw68 (Guo et al.,1992), HLA-A2 (Saper et al.,1991) and H-2K^b (Fremont et al.,1992 and Zhang et al.,1992). The peptides bound to the same class I molecule were found to share sequence motifs which must complement the binding pockets in the class I molecule in question. Each peptide motif has anchoring positions and these also vary depending on the class I molecule involved. It was reported that these positions are 5 and 9 for H-2D^b molecules, and at positions 2 and 9 for H-2K^d and HLA-A2.1 molecules or 5 (or 6) and 8 (or 9) for H-2K^b molecules. The HLA-B27 molecule has

a binding motif that is constrained at position 2 followed by positions 1 and 9 and to a lesser extent position 3. In addition, it was found that the C-terminal residues are always hydrophobic. These restricted pockets in the antigen binding groove are specific for all the members of a family of amino acids (hydrophobic, positively charged...etc.). However, there is usually some preference for one amino acid out of each family (Falk et al.,1991; Madden et al.,1991; Jardetsky et al.,1991; Hunt et al.,1992a; Fremont et al.,1992). The residues not involved in anchoring the antigenic peptide to the groove all have the potential of being exposed enough to interact with the TcR (Madden et al.,1991). The surface of the MHC class I molecule recognised by the TcR is thought to be of similar size to that of a protein antigen that is recognised by the Fab fragment of an antibody approximately 7-9nm^2. This stems from the resemblance between the TcR and the Fab fragment (Davis and Bjorkman,1988; Clothia et al.,1988; Davies,1990). However, Fremont and co-workers (1992), working on the H-2K^b molecule, reported that only a few of the peptide side chains are significantly exposed after binding and in ideal situations they may only contribute some 1-3nm^2 of the surface to the TcR-MHC class I interface. Thus, if the analogy with antibodies holds true, the MHC class I molecule will constitute at least two thirds of what the TcR recognises. A model of the peptide/MHC class I/TcR interaction is given in Figure 6. van Bleek and Nathenson (1991) suggested that it is the polymorphism at the antigen binding groove that exerts a selection pressure and determines the kind of peptides that can bind to a particular class I molecule. Furthermore, Perarnau and co-workers (1990), reported that structural polymorphism of mouse β_2m also restricts the presentation of peptides.

FIGURE 1.6
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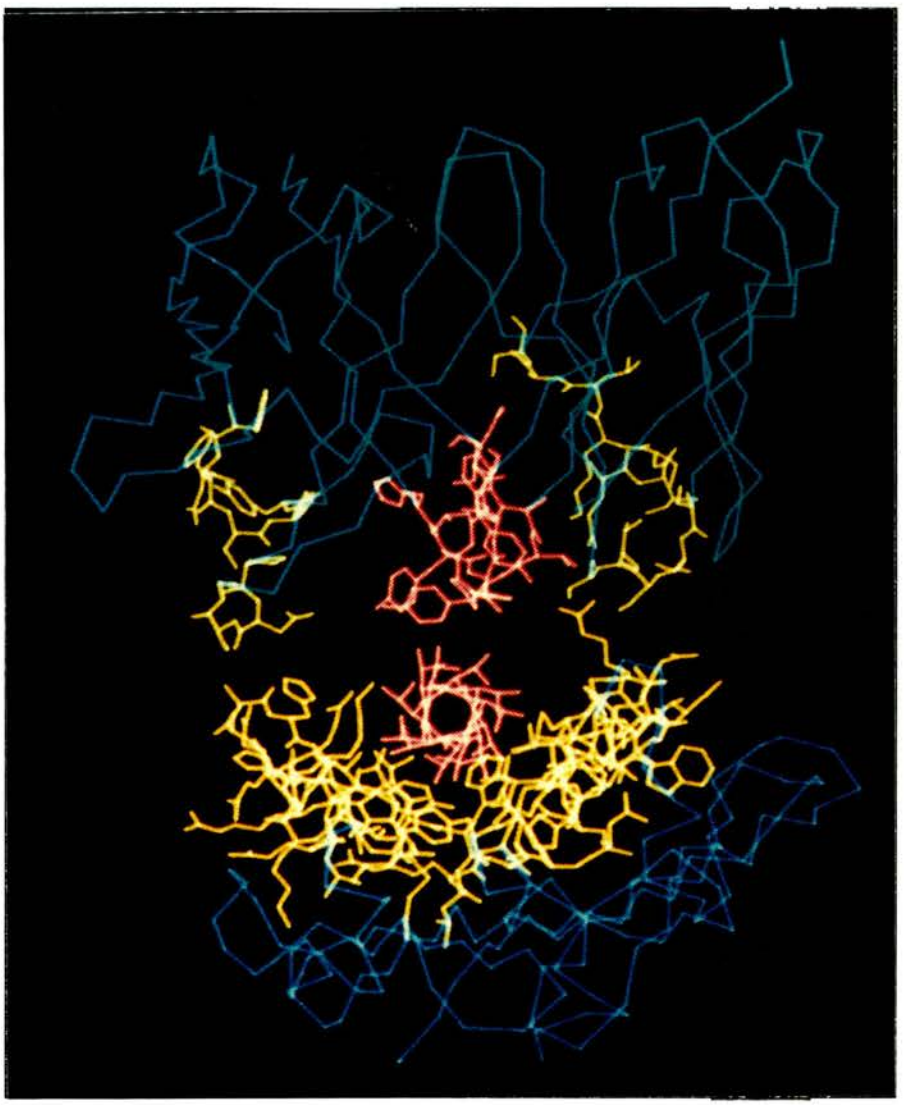
A MODEL FOR PEPTIDE/MHC CLASS I/TcR INTERACTION.

The TcR complex (top of the figure), here represented by a Fab molecule, is bound to an MHC/Peptide complex (bottom of the figure). The colour codes for the MHC/Peptide complex are the same as in figure 5. In this model most of the interaction at the interface between the two complexes is between the peptide and the Tcr and not between the MHC molecule and the TcR as suggested by Fremont and co-workers (1992).



Position 85 was found to be critical for peptide binding. This residue is not a contact residue between β_2m and the floor of the groove (Garret et al.,1989). The restricting effect could arise through either intermolecular interactions between distantly related residues or through species-associated three-dimensional polymorphism of class I molecules. Alternatively, one might consider that the class I molecules are flexible and can adopt configurations other than the ones identified by crystallography which might be critical for the binding of peptide. This limited effect, when combined with the extensive polymorphism of class I heavy chains, would result in the significant enlargement of the species repertoire of peptides presented by class I molecules. Mice express seven different β_2m alleles, humans on the other hand express only one (Perarnau et al.,1990). In addition to the contributions of both heavy and light chains in selecting the peptides that would bind a particular class I molecule, it was recently reported that polymorphism in the major histocompatibility complex-encoded peptide transporter genes is also associated with shaping the range of bound peptides (Powis et al.,1992).

ii: Class II presentation:

Work by Rudensky and co-workers (1991) and Hunt and co-workers (1992b) suggested that, unlike the antigen binding groove of class I molecules, the groove of the class II molecules could be open at one or either end to allow the accommodation of larger peptides; 13-18 amino acids long. The ability of unrelated peptides to inhibit the binding of one another to the same class II molecules suggests that class II

molecules have a single peptide binding site (Buus et al.,1987). Sette and co-workers (1988) following observations by Rothbard (1986), showed that the majority of peptides from Ovalbumin, Myoglobin and Haemagglutonin that bind to I-A^d molecules share a six residue region of the bound peptide that always consisted of the following sequence for the four middle amino acids: polar (or glycine)-hydrophobic-hydrophobic-charged (or glycine). These were HAAH, HVLH for ovalbumin and myoglobin respectively and three of the four from Haemagglutonin were TAA. Hunt and co-workers (1992) reported that the crucial part of the peptides binding to I-A^d is contained within a ten-residue sequence QMVRTAAEVA. This motif determines the capacity of a peptide to interact with a particular MHC molecule perhaps by allowing the peptide to assume the correct conformation for binding. Further work indicated that most peptides capable of binding I-A^d and I-E^d molecules share structural motifs similar to the one described above. The definition of these motifs was found to be broad, and at any given position at least three, and usually more, amino acids are compatible with good binding. This broad spectrum of binding enables the small number of class II specificities to bind a larger number of peptide antigens (Sette et al.,1989; Rothbard and Taylor,1988). The inability to determine similar motifs in a number of peptides that bind these MHC molecules was attributed to the possibility that some of these peptides assume different conformations which might require distinct structural motifs. Indeed, Kurata and Berzofsky (1990) reported that the same peptide can bind the same class II molecule (in this case I-E^d) in more than one way. They also reported that a given residue within a peptide may not have a unique function of always interacting with the MHC molecule or TcR, but may change from

one role to the other as it is presented to different T-cells. Despite this broad specificity, such motifs can be used successfully to predict peptides that are capable of interacting with a given class II^d molecule. Such predictions, whether involving class I or class II molecules, could be of considerable interest in an attempt to select synthetic vaccine candidates to identify possible autoantigens in self molecules, and to select strong binders to MHC molecules that might function as specific inhibitors of MHC-associated autoimmune disease (Sette et al.,1989).

1.5. GENOMIC ORGANISATION OF THE MHC:

1.5.1. The Human and Murine MHCs (HLA and H-2):

The human MHC is located on chromosome 6 at 6p21.1 to 6p21.3 covering a length of DNA of approximately 4Mb. It is 2-3 times the size of the H-2 system which is located on chromosome 17 of the murine genome (Klein, 1975). The general organisation of the different classes of MHC genes relative to each other differs in both species. In mouse the class II and class III loci are located between the class I loci. In the HLA system the class II region is proximal to the centromere followed by the class III region with the class I cluster of genes closer to a telomeric position (Howard, 1987; Trowsdale et al.,1991). The class I genes in both species constitute a multigene family. The HLA system contains up to 20 class I genes, pseudogene and gene fragments (Jordan et al.,1985; Koller et al.,1987 and Srivastava et al.,1987). The number of class I genes present in the H-2 system ranges from 26-33 depending on the haplotype of the animal (Steinmetz et al.,1982; Hammerling et al.,1985; Fisher et

al.,1985 and Weiss et al.,1984). The murine class I genes are located at four encoded loci H-2K, D, L (class Ia loci) and the Qa/TLa region (class Ib loci). Each of these loci is defined by serological analysis of recombinant inbred mouse strains. The K, D and to a lesser extent L loci are highly polymorphic and are expressed on most nucleated cells. The Qa and TLa genes are considerably less polymorphic and exhibit a limited tissue distribution. Moreover, their encoded products have a wide range of molecular weights ranging from 30,000-50,000 (Flaherty et al.,1990). The class Ia loci in humans are HLA-A, B and C (van Rood et al.,1981). Studies by Orr and DeMars (1983), Koller and co-workers (1989) as well as Carrol et al.(1987), Dunham et al. (1987) and Lawrence et al.(1987), using techniques such as analysis of recombination within informative pedigrees, induced HLA-loss mutants and, pulsed field gel electrophoresis (PFGE), predicted that the HLA class I cluster spans approximately 2000 Kilobase (kb). The centromeric boundary being HLA-B and the telomeric boundary not precisely defined. The HLA-B and C loci are most closely linked and appear to be about 85 kb apart (Bronson et al.,1991). The HLA-A locus is at the telomeric end of the system. This subregion has a number of genes with similar properties to genes of the Qa/TLa region of the murine MHC, namely HLA-E, F and G. The function of these class Ib molecules in both mice and humans has not been positively identified (Kress et al.,1983; Flaherty et al.,1990). They have the potential to carry out some of the non-immunological functions suggested for the class I glycoproteins, including a role in adhesion (Zeleny et al.,1978), and contact inhibition (Curtis and Rooney,1979) and expression of hormone receptors (Edidin,1988). They might also have immunological functions as antigen presenters, it was suggested that

they might act as restriction elements for invariant antigens such as the endogenously expressed embryonal and differentiation antigens (Ostrand-Rosenberg et al.,1989). Stress proteins marking damaged cells for possible destruction represent another class of potential endogenous antigen (Asarnow et al.,1988). Another category is carbohydrates, or other unusual nonpeptide antigens derived from bacteria and fungi. During ontogeny the γδ T cells are coordinately expressed with some of the known Qa/Tla genes. In adult mice the great majority of these cells are found in skin, gut and lung; hence the proposed function of γδ T cells and the Qa/Tla molecules function as restriction elements in the immune responses against pathogens in these tissues (Strominger,1989). Additionally, some class Ib molecules (Qa-1^b) are recognised by αβ T cells in TAP2 deficient cells indicating that they not only have the ability to interact with these cells, but additionally that their acquisition of peptide antigen might be TAP-independent (Aldrich et al.,1992). A recent report suggested that the molecule Qa-2 does act as a peptide receptor with ligand specificities (similar to but more stringent than that of class Ia molecules (Rotzschke et al.,1993).

The class II molecules are encoded by genes in the same complex in both species. The HLA-D complex on chromosome 6 and the H-2I complex on chromosome 17. This was mainly established by mapping genomic clones in cosmid and phage vectors. These genes encode a two-subunit heterodimer with α and β chains. It was established that related α and β genes (where both identified) lie close to each other. Most of the α/β gene pairs are disposed tail to tail or (3'-to-3'). The DP subregion in the HLA system is an exception with a head to head arrangement (5'-to-5') for the α/β gene pairs (Okada et al.,1985). Three families of class II genes have

been identified in the HLA system, HLA-DP, DQ and DR (Trowsdale et al.,1984; Okada et al.,1985 and Gustafssen et al.,1987). However, other class II loci might exist. HLA-DNA and HLA-DOB are genes encoding α and β chain sequences respectively, which are different from those encoded by the DP, DQ and DR families (Trowsdale and Kelly,1985; Inoko et al.,1985 and Tonnelle et al.,1985). The H-2I region on the other hand has two defined gene families I-A and I-E. The I-A molecules are considered to be homologues of the molecules encoded by the HLA-DQ genes, and I-E being homologues to HLA-DR (both on sequence similarities) (Widera and Flavell,1985). It was reported recently that a cluster of non-class II genes are located centromeric to DP (Hanson et al.,1991). A series of Really Interesting Novel Genes (RING3,6,7,12,4,9,10 and 11 respectively) lie between HLA-DNA and HLA-DOB genes (Hanson et al.,1991; Kelly et al.,1991a; Robertson,1991; Trowsdale et al.,1991 and Glynne et al.,1991). RING3 has no obvious function in the immune system, RINGs 6 and 7 are highly related to the class II α and β sequences and are candidates to becoming the fourth and fifth class II loci HLA-DMA and DMB respectively (Kelly et al.,1991b; Trowsdale et al.,1991b). RINGs 10 and 12 (LMP-2 and LMP-7 respectively) are thought to encode components of the antigen processing pathway for class I molecules (Glynne et al.,1991; Kelly et al.,1991b). Finally, RINGs 4 and 11 (TAP1 and TAP2 respectively) are thought to encode the transporter molecules involved in translocating antigenic peptides into the ER lumen in order to be coupled to class I molecules (Kelly et al.,1992). The genomic organisation of the HLA system is shown in Figure 7.

FIGURE 1.7
Facing page

THE ORGANISATION OF THE HUMAN MHC COMPLEX.

The map of the human MHC complex including the genes encoding for the class I, class II, class III, transporter and LMP molecules.

1.5.2. The Organisation of the Bovine MHC (BoLA):

The BoLA system has been tentatively localised on the short arm of chromosome 23 bands q13-23 by in situ hybridisation using a cloned porcine class I gene as a probe (Fries et al.,1986).

The class I molecules in cattle have so far only been analysed by serology using antisera raised from calf-dam reciprocal immunisations and alloreactive cytotoxic T-cells (Spooner et al.,1979; Oliver et al.,1981). The allo-antisera are then absorbed against a random panel of cells to reduce their cross-reactivity, and produce "operationally monospecific" allo-antisera that are thought to recognise a single BoLA specificity (Spooner et al.,1980). These allo-antisera, whether against class I or class II are then employed in microcytotoxicity assays. Operationally monospecific allo-antisera, raised against the bovine class II molecules in BoLA class I matched animals, are produced in much the same way as described for the class I allo-antisera, they are however, much more laborious and time consuming to produce, with some class II molecules appearing to be more immunogenic than others (Arriens et al.,1991; Williams et al.,1991). According to the latest workshop, it is thought that there are around 50 class I alleles most of which are encoded by a single highly polymorphic class I locus termed the BoLA-A (Bernoco et al.,1991). The accuracy of the serological typing in these workshops is assessed by the degree of agreement in antigen detection between different laboratories. It should be noted at this point that most of the animals tested and in the BoLA workshops have a similar genetic background (Bos. taurus), with only 7.7% of the total number of animals in the third

workshop representing Bos indicus and only 6.6% of the total were B. taurus * B. indicus crosses (Bull et al.,1989). This, when coupled with the fact that most of the allo-antisera are produced in B. taurus animals, draws the attention to the possible bias that this may incur on the results observed.

Several monoclonal antibodies raised against class I antigens from other species have the ability to detect non-polymorphic determinants on bovine cells (Brodsky et al.,1981; Chardon et al.,1983). These could be the products of less polymorphic class Ia loci such as HLA-C and H-2L or the class Ib products.

It was recently reported that the bovine class I MHC region is estimated to span 1650kb, with 770kb being a minimum estimated size (Bensaid et al.,1991b). The rare-cutter restriction enzyme SfiI cleaves the class I complex (from a single animal) into six fragments. On the assumption that each BoLA class I gene is 5kb, and that the probability of there being an SfiI site in any given 5kb fragment of DNA is low. it is probable that every such fragment contains at least one class I gene as determined by hybridisation with cDNA probes. This is exemplified by the finding that two distinct 3'UT regions are transcribed by the same SfiI fragment of 250kb. Thus, two tightly linked loci are present within a single fragment. These, when added to the genes contained in the five remaining fragments gives the number of class I loci in the bovine MHC to be at least seven. However, the number of functional loci to date is thought to be two relying on evidence from biochemical as well as DNA sequencing and transfection studies (Ennis et al.,1988; Toye et al.,1990; Bensaid et al.,1991a; Joosten et al.,1992; Ellis et al.,1993). A tentative map of the BoLA class I encoding region is given in figure 8.

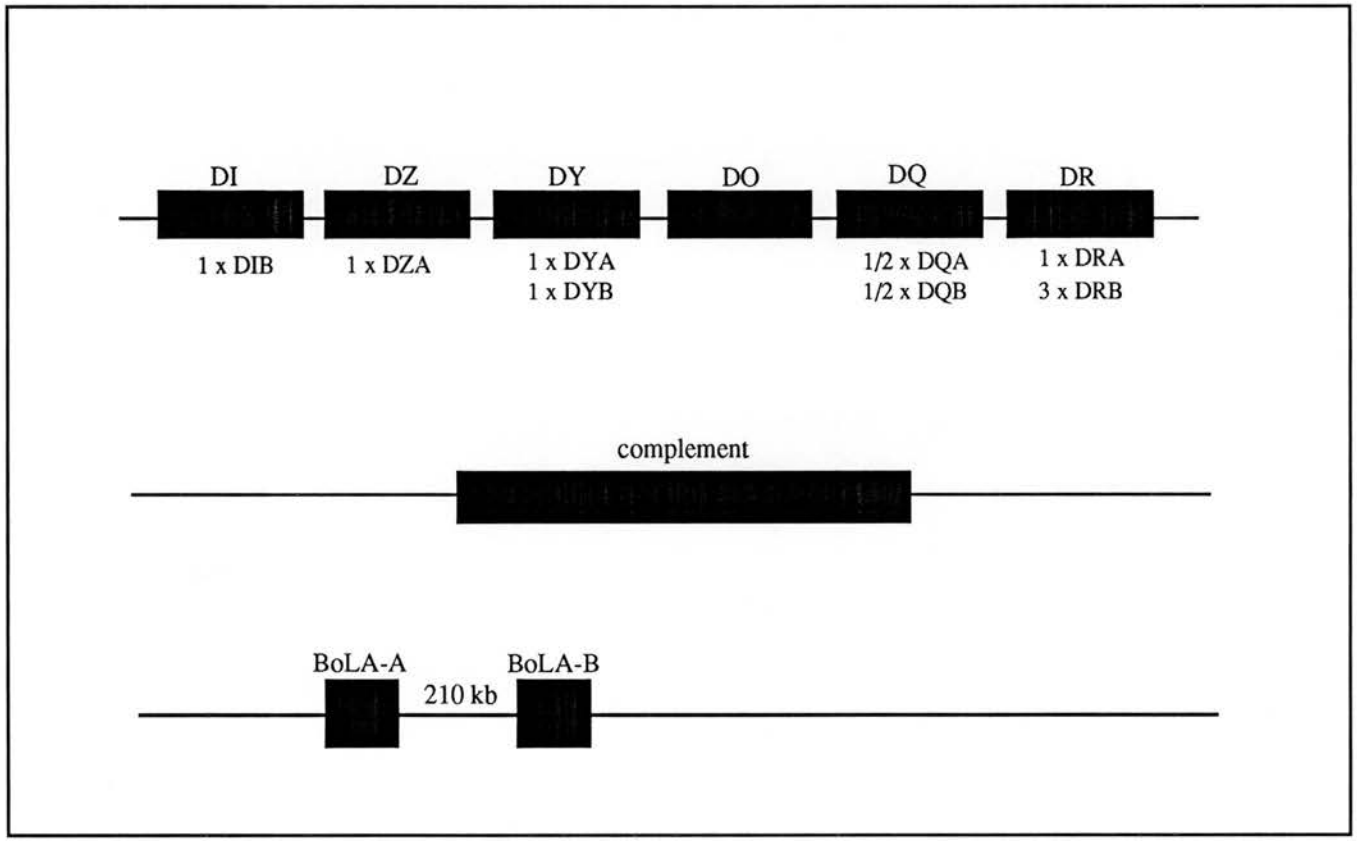
The existence of a class II encoding region in the BoLA system, BoLA-D, has been reported by a number of groups (Spooner et al.,1978; Usinger et al.,1977). Serological methods of studying the complex were employed but proved to have little reproducibility (Usinger et al.,1981). The region is divided into a number of subregions DQ, DR, DO, DZ and DY. Human probes for class I and class II genes were used in RFLP studies to analyse bovine DNA. The results suggested that the numbers of genes within the BoLA system are similar to that in humans, and shows considerable polymorphism at the DNA level. The BoLA-D complex is thought to encode one DRA and three DRB genes one of which was found to be a pseudogene (Groenen et al.,1990), one to two DQA and DQB genes, one DZA gene, one DYA and one DYB. A recent addition to the family was a β-chain encoding gene called DIB (Andersson and Rask, 1988; Andersson et al.,1988; van der Poel et al.,1990; Stone and Muggli-Cockett, 1990). Other results by Andersson et al. (1986a,b) and Vaiman et al. (1986) involving the use of RFLP techniques to analyse bovine genomic DNA using human cDNA probes for the genes of the DR and DQ regions, suggested a variability in the number of class II genes between different haplotypes. Such polymorphism in the number of genes of the DR region has also been reported in the HLA system (Bohem et al.,1985).

Serologically determined BoLA class I and BoLA-DQB types determined using RFLP in Norwegian cattle showed statistically significant strong linkage disequilibrium between the loci encoding these specificities at the population level

FIGURE 1.8
Facing page

A TENTATIVE MAP OF THE BoLA CLASS I ENCODING REGION.

Class I genes that are known to encode expressed products are shown in red. Other class I loci are shown in blue.


DI
DZ
DY
DO
DQ
DR
1 x DIB
1 x DZA
1 x DYA
1 x DYB
1/2 x DQA
1/2 x DQB
1 x DRA
3 x DRB
complement
BoLA-A
210 kb
BoLA-B

(Vage et al.,1992). These results further augment previous reports addressing the same question (Lindberg and Andersson,1988; Mackie and Stear,1990). Lindberg and Andersson (1988) estimated the recombination frequency between the bovine class I and DQ genes to be less than 6.4%, such close genetic linkage may contribute to the linkage disequilibria observed.

General knowledge about the genomic organisation of the BoLA system falls far short of its equivalents in humans and mice. The use of more extensive studies and more advanced techniques is important if more progress is to be made. However, it is a wide belief that the genetic organisation of BoLA-D and the bovine MHC in general is closer to its human counterpart than to that of the mouse (Womack and Moll, 1986; Skow et al.,1988; Groenen et al.,1989; Bensaid et al.,1991b).

1.6. MHC MOLECULES AND DISEASE ASSOCIATIONS:

The MHC class I and class II molecules are specific restriction molecules in antigen presentation. Hence, the presence of a certain MHC molecule could be used as a marker of susceptibility to different pathogens. A better understanding of these associations would give a better chance of predicting the outcome of a disease and will also aid the design of better therapies. A review of the most important associations between MHC molecules and disease is given below.

1.6.1. HLA and disease:

i. Associations with infectious disease:

The human immunodeficiency virus (HIV) has probably been the most extensively studied pathogen in the past few years. The most striking feature of this virus is its ability to persist regardless of the immune system of the host. A recent study, Phillips et al. (1991) defined a single HLA-B27-restricted epitope and three HLA-B8-restricted antigenic epitopes in HIV gag. Analysis of these epitopes revealed that amino acid variation in HIV gag tend to accumulate in or near these $CD8^+$ T cells epitopes, therefore, suggesting that the site of this genetic variation in the virus would influence the ability of the immune system to recognise it, and that this variation is a result of selection pressure by the HLA class I haplotype. From the observation of the relatively high frequency of HIV a.a. mutations observed in HLA-B8 patients, compared with the HLA-B27 patients, it was suggested that the epitope presented by this molecule (HLA-B8) tolerates variation and that such variation does not affect the survival of the virus. The epitope presented by HLA-B27, on the other hand, does not tolerate such variation, probably due to its structural or functional importance to the virus. Thus, patients with HLA-B27 will always have the ability to keep the virus in check whereas individuals with HLA-B8 will be at a disadvantage. This explanation is supported by the observed association of the HLA-A1,B8,DR3 haplotype with poor outcome in HIV infection. The effect of HLA-B27 is not known. HLA-B8 is relatively common in caucasians and could exert an effect on variability of prevalent virus

strains.

In a case study of malaria (Plasmodium falciparum) in West African children it was found that the presence of HLA class I type Bw53 is significantly decreased (15.7%) among individuals with severe malaria. This was determined through serological typing. To make sure that the association observed was not by chance, HLA-Bw53 was isolated and cloned and a PCR dependent method of typing was developed to study the differences in its frequency between severe cases and mild controls. The frequency of the allele was found to be 16.9% among cases of severe malaria and 25.4% among mild controls, an association which was statistically significant (P=0.008) and which confirmed the results of serology. The HLA class II haplotypes in the population were studied using restriction fragment length polymorphism (RFLP). The haplotype DRw13 and DQw1 showed significant differences between children with severe malarial anaemia and the mild controls. DNA sequencing and T-cell assays revealed that the DRw13 allele is in fact the DRB1*1302 subtype. In common with HLA-Bw53 the frequency of this allele in severe malarial anaemia cases is much less than in mild controls, indicating that it is a protective rather than a susceptibility haplotype. The DRB1*1302 was found to be linked to the DQB1*0501. The role of HLA-B53 in protection to severe malaria was investigated by sequencing the peptides eluted from this molecule, followed by screening of candidate epitopes from pre-erythrocyte-stage antigens of Plasmodium falciparum in biochemical and cellular assays. Among malaria-immune Africans, HLA-B53 restricted $CD8^+$ T-cells recognise a conserved nonamer peptide from liver stage-specific antigen-1 (LSA-1) with no restricted epitopes from other antigens (Hill et al.,1992). The class

II molecules, on the other hand, are thought to be involved in the presentation of a particular epitope on a blood-stage parasite antigen to $CD4^+$ T cells, leading to more rapid parasite clearance. This study gave the clearest evidence yet that a lethal infectious pathogen is influencing the evolution of MHC polymorphism by selection pressure (Hill et al.,1991).

Other studies have indicated that the persistence of several RNA and DNA viruses in the neural cells is due to the lack of sufficient expression of class I molecules which is essential for the proper function of $CD8^+$ T cells in immune surveillance mechanism responsible for identifying infected cells in the body, once again pointing to the importance of class I presentation for the combat of such diseases (Joly et al.,1991).

ii. Associations with autoimmune diseases:

MHC class I-associated autoimmune diseases are a small group (compared to the class II -associated ones) and fall in two main categories: (i) the HLA-B27-related spondyloarthropathies, including ankylosing spondylitis, Reiter's syndrome, and reactive arthropathy, and (ii) psoriasis vulgaris, which is associated with HLA-B13,B16 and B17.

HLA-B27 has a strong association with Ankylosing Spondylitis, particularly in Caucasians (Yu et al.,1989). This association may be based on molecular mimicry. Such mimicry arises through a number of amino acid and sequence homologies that have been found between HLA molecules and putative pathogens such as Klebsiella

pneumoniae. Consequently molecular similarities between K. pneumoniae and B27 may be important, such as the six-amino acid sequence shared between HLA-B27 (72-77) and a segment of K. pneumoniae nitrogenase (188-193). The likeliness of this occurring by chance is 1:64,000,000 (Yu et al.,1989).

The involvement of class II molecules in autoimmune disease is much better understood. The HLA-DQ locus was first thought to be associated with type I diabetes (IDDM) after studies by Todd and co-workers (1987) using Restriction Fragment Length Polymorphism (RFLP). Strong confirmation of the proposed association came from ethnic studies. Two haplotypes were identified to be associated with disease susceptibility in Afro-Caribbean populations which are neutral in Caucasian populations. These haplotypes were DR7 and DR9. In Africans, both of these haplotypes have new RFLP patterns around HLA-DQ (Fletcher et al.,1988a). Sequencing studies established that the DR7 haplotype, associated with susceptibility in Africans differs from that in Caucasians at the DQα locus only. The DQα locus on this haplotype in Africans is identical to that on the DQ haplotype in Caucasians, a strong susceptibility haplotype. The DR7 haplotype in Caucasians is neutral for diabetes susceptibility, an unexpected finding as it shares its DQβ locus with the DR3 haplotype. The DQα on the Caucasian DR7 haplotype must be responsible for the neutral effect of this haplotype, and when it is replaced with a susceptibility-associated DQα in the African population, the DR7 haplotype becomes a susceptibility haplotype (Todd et al.,1989). Other work on ethnic groups has confirmed the association of DQ with IDDM (Fletcher et al.,1988b). Todd and co-workers (1987) reported that an aspartic acid at position 57 of the DQβ correlates with resistance to IDDM. However,

studies by Tait and co-workers (1988) and Sheehy and co-workers (1989) suggested that in some cases extended haplotype associations cannot be accounted for by DQ alone, implying that there is an additional DR effect or that another gene is involved.

Specific candidate genes have been identified which account for the association between HLA and susceptibility to Rheumatoid Arthritis (RA), namely the Dw4 and Dw14 genes of the DRβ1 locus (Nepom et al.,1987). These two genes are subtypes of HLA-DR4. Nepom and co-workers (1989) reported that not all patients with RA carry a Dw4 or Dw14 gene. However, the majority of DR4 non-carriers expressed an identical Dw14-like nucleotide sequence in a portion of the DRβ gene. A single oligonucleotide probe based on this sequence identified not only DR4 susceptibility genes, but also DRβ genes in most DR4-negative patients with RA. The nucleotide sequence recognised by this probe encodes part of the DRβ polypeptide's first domain, centred on codon 71, a residue directly implicated in T cell recognition of this allele (Seyfeird et al.,1988). These findings indicate that a shared nucleotide sequence contributes shared epitopes among different HLA genes, which would explain the apparent genetic heterogeneity of RA patients. Therefore, in RA, this fragment of nucleotide sequence within the DRβ1 gene, rather than any single allele, appears to be the primary susceptibility determinant of the disease.

The class II association with Multiple Sclerosis (MS) is with HLA-DR2 in Caucasian individuals, although other ethnic studies have indicated that there are increased frequencies of DR4 and DRw6. Comparison of the sequences of these three molecules has revealed a sequence in DQβ that may be associated with MS (Vartdal et al.,1989). Studies by Heard and co-workers (1989) have suggested that a haplotype

other than DR2 may contribute to MS susceptibility.

Whether it is a class I or class II association, susceptibility to autoimmune disease appears to be multifactorial, with environmental factors also playing a major role. In other words autoimmune responses could be initially triggered by an antigenically similar, cross-reacting environmental pathogen. This phenomenon is often referred to as molecular mimicry. Susceptibility to autoimmune disease is also polygenic. Because of the multifactoriality and the polygeneity, autoimmune susceptibility is not inherited in a simple Mendelian fashion (Sinha et al.,1990; Benjamin and Parham, 1990).

1.6.2. BoLA and Disease:

One of the main reasons for undertaking studies of the BoLA system is that it may provide a means for selecting more productive or disease resistant cattle, by identifying BoLA types which are associated with good characteristics, or selecting against those associated with bad traits.

It has been shown that different breeds of cattle exhibit different frequencies of BoLA serologic specificities (Oliver et al.,1981; Spooner et al.,1987; Kemp et al.,1988 and Stear et al.,1988a). Differences between breeds might indicate which specificities should be studied further to identify possible associations with disease, growth rate and reproductive performance. The following is a description of what is known about the BoLA molecules, both class I and class II, and their association with disease.

The tick-borne protozoan parasite Theileria parva causes an acute, usually fatal, disease of cattle known as East Coast Fever. This results in major economic losses throughout east and Central Africa. It was shown by Goddeeris et al. (1986) that $CD8^+$ T cells which are important in mediating immunity to T. parva are restricted by class I MHC molecules (serologic specificity KN104), and are parasite strain specific. A more recent study by Goddeeris et al. (1990) further implicates the serological specificities w7 and w6, the latter appeared to be more dominant in the presentation of the parasite to the T-cells in animals showing both class I specificities. w18 was also seen to have a restricting effect. Bovine class I molecules have also been implicated in the restriction of $CD8^+$ T cells responses to bovine herpes virus-1. However, no clear cut associations with particular serotypes were found (Splitter et al.,1988). It was also recently reported that class I molecule compatibility between the calf and dam is involved in the aetiology of bovine retained placenta (Joosten et al.,1991). Perhaps the most striking association between BoLA class I and disease to date, is the report that the BoLA w8 serotype is significantly associated ($p<0.001$) with chronic posterior spinal paresis (PSP), a form of osteophytosis/ankylosing spondylitis in Holstein bulls. The same serotype showed statistically significant association with PSP ($p<0.0015$) in a population of unrelated bulls, it also showed significant associations ($p<0.0008$) with PSP in a family of 13 animals sired by a bull with the serological type w8/w20. All the individuals inheriting the w8 haplotype had PSP, whilst all the healthy siblings inherited the w20 haplotype (Park et al.,1993).

It was suggested by Glass et al. (1990a,b) that there are definable bovine class II haplotypes which are functional in antigen presentation and possibly also for Ir

(immune response) gene effects. In a recent study, it was found that foot and mouth virus (FMDV) peptides could be restricted by particular MHC class II molecules. Animals with class II types EDF 5 and 11 confer low responsiveness to the putative peptide vaccine FMDV15. EDF types 2,3,4,6 and 7, on the other hand, were able to present this peptide (Glass et al.,1991).

Various other associations between BoLA (mainly class I molecules) and a number of diseases have been reported. However, most of these studies relied on statistical analysis and not on any immunological or biochemical data. A description of these is given below.

The effect of the BoLA system on susceptibility to mastitis was studied in Norwegian cattle, and it was found that the BoLA type w2 was associated with resistance, while w16 was associated with susceptibility (Solbu et al.,1982; Spooner et al.,1988). Larsen and co-workers in 1985 also indicated that W16 could have an effect, through an indirect association with the M blood group. w2 in Icelandic cattle, on the other hand, is associated with susceptibility to mastitis, however, this specificity is thought to be different from the one reported above (Oddgeirsson et al.,1988).

In a study on a herd of Holstein Friesians it was shown that genes closely linked, probably within the BoLA complex, are associated with the subclinical progression of the bovine leukaemia virus (BLV). There is a significant negative association between the w14 specificity and the presence of antibodies to BLV-gp51. The same study showed that susceptibility to the polyclonal expansion of BLV infected B-cells in BLV infected hosts was associated with either w1 or w12 specificities (Lewin et al.,1988). In addition, Lewin and Bernoco (1986) demonstrated

that in a herd of Shorthorn cattle, resistance to B-cell proliferation in BLV-infected cows segregated with w4 and w7-bearing haplotypes, and susceptibility with w26 bearing haplotypes. Studies by Stear et al. (1988b) and Hines et al. (1985) associated w6 with susceptibility to Persistant Lymphocytosis (PL) (another form of infection with BLV). Evidence was given by Hines and co-workers (1985) that susceptibility to (PL) and lymphosarcoma is associated with w15 in Holsteins and w31 in Jerseys. These results excluded the possibility of a direct effect of BoLA specificities, but suggested the existence of a linked effect. Recently, the class II BoLA allele, or a very closely linked gene, was shown to be associated with resistance to PL (van Eijk et al.,1992). Other associations with tick infestation, cancer eye, worms and even weight gain and milk production were reported (see Stear et al.,1984, 1988c, Stear, 1988 and Hines et al.,1986).

1.7. THE STUDY OF THE MHC AND VACCINE DESIGN:

A better understanding of the structure and function of the MHC molecules is immensely important to the design of safer, more potent and successful vaccines the ultimate aim in the application of such knowledge. An ideal vaccine would produce complete protection from infection after a single injection, evoke minimal side effects, be readily available, cheap, stable and easy to administer.

Traditional vaccines consist of either live attenuated or killed organisms. Such vaccines have considerable inherent problems some of which are: high restriction to

strain and type specificity due to continuous antigenic variations in viruses and parasites; difficulties in production, storage and delivery; contaminating materials and undesirable side effects. Also, there are still many viral and parasitic diseases for which no effective vaccine exists. Therefore, alternative approaches to vaccine preparation are being sought.

The use of synthetic peptides for vaccination is one alternative which is being explored extensively. It is attractive due to the simplicity of the approach, the information it brings to the molecular understanding of the immune response requirement for protection and the enormous practical advantage that such products could offer. These vaccines usually constitute small peptides carrying epitopes of the organism against which the vaccine is designed. By selecting these epitopes only, it should be possible to exclude others which may elicit deleterious immune responses. Additionally, peptide vaccines are chemically defined, therefore, free of infectious material, and can be readily and cheaply synthesized in unlimited quantities (Arnon and Horwitz,1992). To synthesize a peptide which can elicit an immune response against foreign protein, it is necessary to determine the sequence of amino acids which comprises the necessary immunogenic determinants. The nature of such peptides and the way they are presented to the immune system has recently become clearer. Processed antigenic peptides bind to the class I and II molecules of the immune system only if they have the correct code or motif for the MHC molecules present (Sette et al.,1988; Falk et al.,1991; Madden et al.,1991; Jardetsky et al.,1991; van Bleek and Nathenson,1991; Hunt et al.,1992; Hunt et al.,1992a,b).

These findings have enabled different groups to predict the sequences of peptides that

would bind better than others to the same MHC class I molecule (Falk et al.,1991; Tsomides et al.,1991). Similar predictions in the context of murine class II molecules ($I\text{-}A^d$ and $I\text{-}E^d$) were made by Sette and co-workers (1989).

This type of work is invaluable in its potential application to peptide vaccine design. However, in order to be able to direct a certain peptide to elicit a humoral or a cell-mediated immune response and protective immunity it is vital to find the delivery system which would introduce these peptides into the correct presentation pathway. Such delivery systems, whether chemical or biological, are currently being designed and a review of some is given below.

Immunisation with free peptide could in some cases lead to protective immunity. Kast and co-workers (1991) and Schulz and co-workers (1991), working on, nucleoprotein T cell epitopes from Sendai virus and Lymphocytic Choriomeningitis Virus (LCMV) respectively, reported that immunisations with peptides from these proteins elicited $CD8^+$ T cell responses in mice.

Schild and co-workers (1991) reported that coupling various peptides of the influenza virus to the lipopeptide tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P_3CSS) was successful in inducing virus-specific $CD8^+$ T lymphocytes. This response was comparable with the priming efficiency seen with the infectious virus itself and was better than the response induced by the peptide alone. Deres and co-workers (1989) reported that the peptide corresponding to the sequence comprising amino acids 147-158 of the influenza nucleoprotein, when covalently linked to P_3CSS, induced high affinity $CD8^+$ T cells as does the infectious virus.

Another method for the development of completely synthetic products

containing both antigen and carrier is the multiple antigen peptide (MAP) system. The surface of this macromolecule consists of multiple clusters of the antigen epitope with a small oligolysine core at the centre. This system has the advantage of being completely chemically defined, containing either multiple copies of a single epitope or different antigenic peptides attached to the same macromolecule (Tam,1988).

Synthetic peptides from the V3 region of the gp120 protein of IIIB, RF and MN HIV-1 isolates were used as the antigen. MAP consisting of various chain lengths, from 11-24 residues, were prepared in a monoepitopic configuration containing four repeats of each individual peptide. They were synthesized in parallel in a diepitope configuration adding at the carboxyl-terminus of the V3 peptides a conserved sequence, known to be a $CD4^+$ T cell epitope of gp120. The antibody response elicited by the monoepitope constructs were measured in three experimental species and was found to be species dependent. Diepitope MAPs were immunogenic in all three species and elicited significantly higher antibody titers than those raised by immunisation with the monoepitope MAPs. These results demonstrated the potential usefulness of a MAP system for the production of an HIV vaccine (Nardelli et al.,1992).

The Foot and Mouth disease virus (FMDV) was one of the earliest viruses in which a synthetic peptide approach was attempted. The MAP approach was employed using the virus-neutralising antibody inducing peptide 141-160 of the VP1 protein. Monomer, tetramer and octamer structures of this peptide were examined. The tetrameric structure was found to give the optimal response, with levels of neutralising antibody comparable with those obtained when whole FMDV was attached to various

carrier proteins (Francis et al.,1991).

The efficiency of the MAP system was demonstrated using parasites. The circumsporozoite protein (a major malaria sporozoite surface antigen) of Plasmodium falciparum, which consists of the four amino acids NANP repeated up to 40 times was used. It has been shown that synthetic and recombinant NANP peptides were immunogenic only in H-2^b mice (Good et al.,1986; del Giudice et al.,1986; Tonga et al.,1986; Lussow et al.,1990). The incorporation of this repeated sequence of the NANP peptide in a MAP system seem to overcome this restriction. This function did not appear to be due to the peculiar assembly of the NANP sequence. In fact, MAPs with repetitive sequences from the same protein taken from other strains of the parasite did not overcome the genetic restriction of the immune response to the linear peptides. This data suggested that the immunogenicity of the P. falciparum NANP repeats can be dramatically changed and increased when the MAP system is used.

Perhaps the best example of a potential recombinant vaccine vehicle is the live attenuated bovine tubercle bacillus BCG (Bacille Calmette-Guerin). This multivaccine vehicle has been the most widely used in vaccines since 1948, with approximately half the worlds population being exposed to it at one time, it can be given at birth, has the ability to sensitize to tuberculoproteins 5-50 years after a single inoculation, it is a potent adjuvant in a variety of species, could be orally administered, heat stable and is inexpensive to produce. Additionally, recombinant BCG was shown to express a number of viral, bacterial and parasitic antigens (Stover et al.,1991).

Hybrid genes containing DNA fragments of HIV-1 Gag, Pol and Env precursors fused to the microbacterial promoter and translation start site hsp70 so that

they completely replace the hsp70 coding sequences, were inserted into pYUB12 (a BCG autonomous replicating plasmid vector). The recombinant vector was introduced into the Pasteur strain M.bovis BCG by electroporation. BALB/c mice intravenously inoculated with this recombinant vector had low but detectable antibody responses demonstrating that the recombinant BCG can elicit humoral responses to foreign proteins produced by bacillus. In addition, mice inoculated with the BCG-HIV Gag recombinant produced cell-mediated immune responses as measured by cytokine production and the cytotoxic activity of spleen cells from these animals (Aldovini and Young,1991).

The above findings when combined with the various features of BCG and the ease with which bacteria can be manipulated makes it possible to tailor a BCG vaccine that would maximise immune responsiveness.

Other bacterial delivery systems, such as attenuated Salmonella typhimurium, have been used to introduce a plasmid encoding the influenza nucleoprotein gene from A/NT/60/68 virus. The results showed that the bacterial vector expressing the viral gene induces both humoral and cell-mediated immune responses. However, only $CD4^+$ virus specific T cells capable of proliferation were readily induced, and in some cases, class II MHC restricted cytotoxicity was detected (Tite et al.,1990). Similar systems have been studied with a variety of recombinant vaccines (Leclerc et al.,1990; Schodel et al.,1990; Newton et al.,1989).

The refinement of these vector systems, together with the accumulating knowledge concerning the way in which peptides are presented to the cells of the immune system by the MHC molecules, can only push us ever closer to finding a

reliable and safe system of peptide vaccines.

It is clear from the above that the study of the MHC molecules in humans as well as in other important species is of great benefit. The better understanding of the functional aspects of these polymorphic products can only come from detailed studies of their basic structure and assembly. As cattle are important economically all over the world, the understanding of the basics of their immune system is a priority so that this knowledge can be used in producing healthier, more productive animals. The following pages will carry a description and analysis of experiments carried out with that ultimate aim in mind.

CHAPTER TWO

Aims

2. AIMS:

Cattle have been part of agricultural everyday life for thousands of years. The first evidence for the domestication of cattle comes from tomb pictures and statues depicting cattle being milked and butchers at work. These findings led archaeologists to conclude that cattle were domesticated between 6000 and 5000 B.C. in Mesopotamia (Friend,1978). Ever since domestication, cattle have spread with the migration of people, and have undergone a great deal of selection and faced a wide variety of pathogens. Of high interest in animal genetics and breeding are the possibilities of enhancing resistance to various disease pathogens. In order to be able to select for healthier animals it is important to have genetic markers, these can have either a direct or indirect effect. The central role of the MHC to the health of the animal and its indirect effects on growth and development makes it of particular interest as a genetic marker for selection studies. In order to fully appreciate the power and influence of the MHC, it is essential to understand its genetic and molecular structure as well as the influence of each of its products.

Most of the knowledge about the MHC of cattle (BoLA), over the first decade of its study has come from serological studies. Over the past five years much has been done to employ more sensitive methods in the study of the BoLA system, although they represent only the first few steps along a very long path towards an understanding of the true complexity of the bovine MHC.

Therefore, this project was designed to use biochemical methods to shed some light on the polymorphism and origins of the molecules expressed by the BoLA class

I encoding region. The serological studies, which represent the vast majority of investigative studies on the BoLA system, have indicated the presence of a single highly polymorphic locus encoding class I molecules. However, other biochemical and molecular biological studies have indicated that the system is much more complex than is observed by serology alone. One biochemical method employed recently to study the BoLA system is 1D-IEF. Results from studies using this technique give highly complex patterns from individual animals, these results contrasted sharply with those obtained from serology. Therefore, experiments were designed to investigate the origin of this complexity and to determine whether it is the product of the post-translational modification of a small number of polypeptides, two in the case of a single heterozygous locus or, on the other hand, products of several distinct class I loci.

The initial hypothesis was that the complexity observed with 1D-IEF is the product of differential post-translational modifications of the products of one or two class I loci. Post-translational modifications that could be responsible for the charge heterogeneity observed include glycosylation and phosphorylation. Alternative splicing of pre-mRNA is a post-transcriptional modification which is usually reflected in the protein product. This mechanism could give rise to isoforms of the same molecule.

The effects of glycosylation were addressed using the enzymes neuraminidase and endoglycosidase F/N-glycosidase F, to treat ^{35}S methionine labelled class I molecules that were immunoprecipitated using monoclonal antibodies. All samples were analysed on SDS-PAGE and 1D-IEF gels.

The effects of phosphorylation were investigated by labelling cells with $^{32}P_i$ and then immunoprecipitating the class I molecules and analysing the samples by SDS-PAGE and 1D-IEF. These samples were compared to others from cells labelled with ^{35}S methionine. Additionally, immunoprecipitated samples from cells labelled with ^{35}S methionine were subjected to enzymatic digestion with potato acid phosphatase and analysed by SDS-PAGE and 1D-IEF.

The possibility of alternative splicing of pre-mRNA having an effect was dealt with indirectly using two-dimensional electrophoretic analysis. Furthermore, to test whether the molecules observed did in fact have structural differences that might be due to differences in their primary structures, partial digests of these molecules were analysed by peptide mapping employing the enzyme endoproteinase Glu-C (V8).

To further understand the origin and expression pattern of the molecules observed, the AAS used in serology were used in the immunoprecipitation of their targets and these samples were compared to those produced by using monoclonal antibodies.

Finally, the available BoLA class I sequences in addition to the HLA-A2 sequence were subjected to structural analysis using the GCG computer software package and the results were compared to what is known about the structure of the class I molecules of the HLA system.

The details of the methods used and the results from the experiments outlined are given in chapters 3 and 4. A discussion of these results and a conclusion are given in chapter 5. The details of the materials, and other information regarding the composition of the bovine allo-antisera are given in the appendix.

CHAPTER THREE

Methods

3. METHODS:

3.1. LYMPHOCYTE PREPARATION AND METABOLIC LABELLING:

The recipies of all buffers and media and information about molecular mass markers and suppliers are given in appendix A1.

3.1.1 Lymphocyte preparation:

Lymphocytes were prepared by standard ficoll/hypaque centrifugation. 20ml heparinised blood (25U/ml) was diluted with an equal volume of HBSS, layered onto 12.5ml ficoll/hypaque (s.g. 1.069) and centrifuged at 1500xg (IEC Centra-8) for 25 mins. at 37°C. The lymphocytes were harvested from the ficoll/plasma interface and washed 3 times with HBSS. The cells are resuspended in 3ml of tissue culture medium (MEM (Gibco)+ 10% foetal calf serum), counted and numbers adjusted to $4.5x10^7$/ml. This is sufficient for 3 immunoprecipitations ($1.5x10^7$ each).

3.1.2 Metabolic radiolabelling:

$4.5x10^7$ cells were centrifuged at 250xg for 10 mins., resuspended in 2.5ml of methionine free medium and incubated at 37°C in an atmosphere of 5% CO_2 for 30 mins.. ^{35}S methionine (15mCi/ml, Amersham) was added to a concentration of 20uCi/ml and the cells were incubated overnight under the same conditions as above.

For phosphorylation, cells are incubated in 1ml of phosphate free buffer for 90 mins., at 37°C then incubated with 80uCi/ml of $^{32}P_i$ (10mCi/ml, Amersham) for 3 hrs at 37°C.

3.2. IMMUNOPRECIPITATION OF BoLA CLASS I MOLECULES:

Labelled cells were centrifuged for 5 mins. at 200xg and washed once by resuspending in 1ml PBS, the sample is then transferred to an Eppendorf tube and centrifuged at 10,000xg for 20 sec.. The pellet was then resuspended in 1ml of TX114 lysis buffer and incubated on ice for 30mins.. The cell lysate was centrifuged at 7000xg for 10 mins. at 4°C. The supernatant was transferred to a new tube and incubated at 37°C for 4 mins.. The detergent/aqueous phases were subsequently separated by centrifugation at 200xg for 4 mins. at 37°C and the aqueous phase was discarded. The labelled lysate was precleared by incubating with 4ul of normal rabbit or mouse serum and incubated on ice for 30mins. with 1ml NET buffer. Pansorbin cells from Staphylococcus aureus (50ul of 1/10 solution in NET buffer, Calbiochem) was added and incubated for 15 mins. (on ice). The samples were then centrifuged and the supernatant is transferred to a fresh tube. The last step was repeated.

330ul of labelled lysate was used for each immunoprecipitation and was incubated with 4ul of MAb (ascitic fluid) or 50ul of AAS on ice for 75mins.. The MHC class I/antibody complexes were subsequently precipitated by incubating with 100ul of Pansorbin cells (for MAb) or 100ul neat Gammabind G (BDH, for AAS) on ice for 30 mins. Preparations were then centrifuged at 5000xg for 4 mins., and the supernatant was discarded. The pellet was then washed x4 in 0.5ml NET buffer. After

final wash, the pellet was resuspended in 100ul of 1U/ml neuraminidase in 50mM/l sodium acetate, pH 5.5 containing 154mM/l NaCl and 9mM/l $CaCl_2$ (Behring) and incubated at 37°C overnight. A further 0.1U of neuraminidase was added and incubated as above for an additional 6 hrs.. The supernatent was removed and the appropriate sample buffer for each application were as follows: The SDS-PAGE sample buffer contained 2% SDS, 20% glycerol, 0.125M Tris pH 6.8, 0.004% bromophenolblue and 5% 2-mercaptoethanol, the samples were boiled for 2mins. before application to the gel. The IEF sample buffer contained 28.5g urea, 1ml ampholine pH range 3.5-10, 1ml 10% NP40 and 2.5ml 2-mercaptoethanol. The samples were incubated at 37°C for 30mins. prior to application to gel.

When the enzymes EndoF and Potato acid phosphatase were used, the samples were incubated with a variety of enzyme concentrations (0.1-0.4U of EndoF and 0.15-0.25U of potato acid phoaphatase) for various lengths of time (3hrs-overnight)(see Figs. 4.5, 4.6 and 4.9).

3.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS:

12% polyacrylamide gels were set using solutions described in appendix A.1. 30ul of sample was added to each well and fractionated by electrophoresis at 200v for 40mins for mini gels using the Mini pII system (BioRad). Larger gels (20x20cm) were run at 70v until samples reach the interface between the stack and resolving gels and then at 150v for 5hrs. The gels were removed, incubated with DMSO twice for

30mins. each and then in PPO/DMSO (250g/l) for 3hrs.. After an additional 30min. incubation in tap water the gels were dried and visualised by autoradiography using Kodak X-OMAT S x-ray film for 7 days at -70°C.

3.4. 1D-ISOELECTRIC FOCUSSING:

Gel solutions (see appendix A.1) were poured into assembeled apparatus and left to set. The bottom spacer and comb were then removed and the wells were rinsed with DH_2O. The bottom reservoir of the IEF apparatus was filled with 20mM H_3PO_4 and the gel assembly was then clipped into position on the IEF apparatus. Care was taken to ensure that the bottom of the gel was in contact with the buffer. Any gaps between the top of the gel assembly and the top reservoir were sealed with 1% agar and petroleum gel. Two wells at either end of the gel were also filled with 1% agar. The samples were incubated with 25-30ul of sample buffer for at least 30 mins. at 37°C. They were then centrifuged at 10,000xg for 4 mins. and loaded onto the gel. The samples were overlaid with 8-10ul of overlay buffer and the samples were further layered with 50mM NaOH as top tank buffer. Gels were run at increasing voltage from 400v-950v until equilibration (overnight). The gels were then removed and treated with DMSO and DMSO/PPO as described in section 3.3. The banding patterns were visualised by autoradiography by incubating at -70°C for 7 days.

3.5. 2D ISOELECTRIC FOCUSSING/SDS-PAGE ELECTROPHORESIS:

3.5.1 First dimension (Mini IEF):

This method was carried out using the BioRad mini 2D cell. The IEF gel solution (29.7g Urea, 8.3ml Acrylamide 30T/1.6C, 3ml Ampholines of which 75% was pH 5-7, 18% pH 3.5-10 and 7% pH7-9, 100ul ammonium persulphate, 50ul TEMED) was allowed to flow gently down the side of the casting tube filling the IEF tubes from bottom to top and the preparation was left to set.

The second dimension gels were prepared as in section 3.3. The capillary tubes were removed from casting tube and rinsed with water. The tubes were connected to sample reservoirs. Samples were prepared as in section 3.4. and were added to the reservoir. Top and bottom buffers were added to the apparatus. The gels were run at 500v for 10 mins. followed by 60 mins. at 1500v, the tubes were removed from holder and attached, to tube gel ejector, partly filled with equilibration buffer.

3.5.2 Second Dimension:

Each IEF tube was layered onto a 12% SDS-PAGE as a second dimension. Electrophoresis was then carried out as described in section 3.3. No agarose was used to hold gels down. 1ul of ^{14}C labelled low molecular mass markers (Sigma) was added to the gel.

3.6. PEPTIDE MAPPING ON THE SECOND DIMENSION:

The procedure is as described in section 3.5. with the following modifications:

a. After removing the 2D equilibration buffer. The tube gel was overlayed with 100ul of V8 protease (Boehringer Mannheim) dilution buffer containing 2ul of enzyme solution. The buffer was then sealed onto the tube gel with 1% agarose.

b. The gel was run until the dye front reached the interface between the stack and the resolving gel, when the procedure was stopped for 20-30 mins. to allow digestion to take place. The procedure was then continued as in section 3.5..

3.7. SEQUENTIAL IMMUNOPRECIPITATION OF BoLA CLASS I MOLECULES:

The procedure here was the same as that described in section 3.2., however, in this case the samples were subjected to three consecutive cycles of immunoprecipitation with W6/32 (8ul each were added per sample of $2x10^6$ cells). That was then followed by one cycle of immunoprecipitation with IL-A88 (10ul). Pellets collected at each stage were treated as described in section 3.4..

3.8. TISSUE CULTURE:

T. annulata transformed bovine T cells (Kind gift from Dr.E.Glass) were cultured in 10ml tissue culture medium (RPMI 1640 (Gibco BRL), 25mM Hepes, 200mM L-glutamine, 10% FCS and 5ml of Penicillin/Streptomycin (Gibco BRL) at 10,000U/ml and 10,000 ug/ml respectively) in 80cm^2 flasks (Nunc). The cells were harvested by incubating with 2ml of 0.02% EDTA for 2mins. at 37°C. The cells are then washed by resuspending in PBS followed by centrifugation at 200xg.

3.9. FACS ANALYSIS:

Cells (10^7/ml) transformed with the intracellular parasite T. annulata were harvested as above and resuspended in FACS medium (RPMI (Gibco), 5% horse serum and 0.2% sodium azide) and kept on ice. 50ul of cell suspension ($5x10^5$ cells/well) was dispensed per well (of 96 well round bottomed plate) followed by another 50ul of optimally diluted MAb and shaken once (using orbital shaker), the preparation was then incubated on ice for 30 mins., shaken once during incubation. The plate was centrifuged at 120xg for 2 mins. and the supernatant was removed and the cells were washed by resuspension in 100ul FACS medium and centrifuged at 120xg for 2mins.. The washing steps were repeated three times. The cells were resuspended in 25ul of fluorescent conjugate (1/100 of FITC-rabbit anti-mouse or rabbit anti-bovine in FACS medium, Nordic Laboratories) and the plate was incubated

on ice for 30 mins.. The preparation was then centrifuged and washed three times in 100ul FACS medium, resuspended in 100ul of FACS medium and analysed using FACscan (Beckton Dickinson). The results were analysed using Consort 30 and Lysis 1 software.

3.10. DEFATTING MILK:

The milk was diluted 1/5 in DH_2O and mixed well. The samples were centrifuged at 10,000xg for 15 secs., the fat adheres to the sides of the tube.

CHAPTER FOUR

Results

4. RESULTS:

4.1. THE BIOCHEMISTRY AND EXPRESSION OF BOVINE MHC CLASS I MOLECULES:

4.1.1. General characteristics:

Bovine class I molecules have been poorly studied when compared to similar molecules from other species. In this chapter, a series of experiments will be described with the objective of answering fundamental questions concerning the biochemistry of the expressed BoLA class I molecules, and their mode of expression.

In all these experiments the class I molecules were immunoprecipitated from ^{35}S methionine metabolically radiolabelled, non-stimulated, bovine peripheral blood lymphocytes (PBL) lysed in 0.5% TX114 buffer. This detergent phase separates at temperatures over 20C, hydrophilic proteins are found in the aqueous phase and integral membrane proteins with an amphiphilic nature, such as class I MHC molecules, are recovered in the detergent phase (Bordier,1981). The samples are then treated with neuraminidase to reduce the charge heterogeneity, and then analysed by 1D-IEF or SDS-PAGE. The immunoprecipitated samples were first analysed for their molecular mass using 12% SDS-PAGE. Figures 4.1, 4.2 show the results from two different gels. In figure 4.1 three monoclonal antibodies recognising different epitopes on the class I molecules were used. MAb W6/32 is a mouse antibody that recognises a monomorphic conformational epitope on the heavy chain of human class Ia and some class Ib (HLA-G) molecules, which cross reacts with bovine class I molecules in a monomorphic fashion (Brodsky et al.,1981). The appearance of this epitope is

FIGURE 4.1
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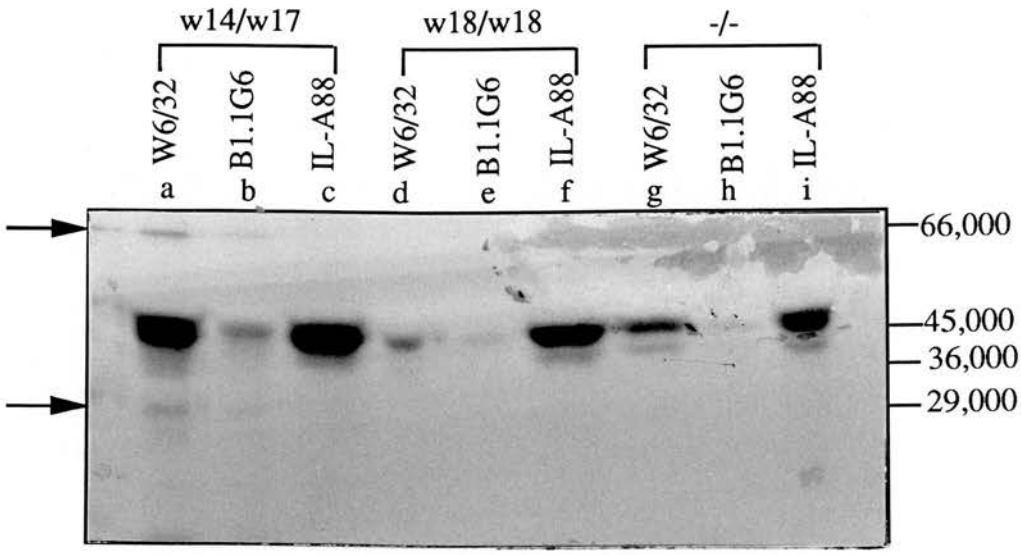
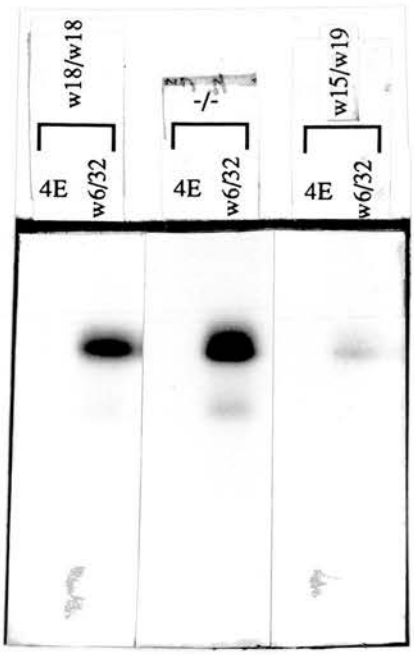
MOLECULAR MASS OF MOLECULES IMMUNOPRECIPITATED WITH MAbs W6/32, B1.1G6 and IL-A88.

Figure 4.1a 12% SDS-PAGE analysis of neuraminidase treated molecules, immunoprecipitated from metabolically radiolabelled lymphocytes from animals with the serotypes w14/w17, w18/w18 and an animal of unknown serotype (-/-).
Lanes a, b and c represent samples from an animal with the serotypes w14/w17, immunoprecipitated with MAbs W6/32, B1.1G6 and IL-A88 respectively. Lanes d, e and f represent samples from an animal with the serotypes, w18/w18, with the same MAbs in the same sequence. Lanes g, h and i represent samples from animal of unknown serotype immunoprecipitated with the same MAbs in an identical sequence to the previous two animals. The molecular mass markers are indicated by the arrows on the right. The arrows to the left of the figure represent spill over from the marker lane. Figure 4.1b represents SDS-PAGE analysis of immunoprecipitations from three animals with MAb W6/32 and the negative control MAb 4E (IgG_{2a}).

(4.1a)


w14/w17
w18/w18
-/-
W6/32
B1.1G6
IL-A88
W6/32
B1.1G6
IL-A88
W6/32
B1.1G6
IL-A88
a
b
c
d
e
f
g
h
i
66,000
45,000
36,000
29,000

(4.1b)


w18/w18
-/-
w15/w19
4E
w6/32
4E
w6/32
4E
w6/32

dependent upon the association of the heavy chain with β_2m (Parham et al.,1979; Jefferies and MacPherson,1987; Shimizu et al.,1988). MAb B1.1G6 is another mouse antibody, which recognises an epitope on the β_2m subunit of human class I molecules (Libeuf et al.,1981), and was a kind gift from Dr.B.Mallison. MAb IL-A88 is a mouse antibody that recognises a monomorphic non-conformational epitope on the bovine class I heavy chain (M.Subash, Personal communication) and was a kind gift from ILRAD. For negative controls see figures 4.1b and 4.3e. Figure 4.2 shows the results of molecular mass determination experiments using bovine allo-antisera (AAS) instead of MAb for the precipitation step. The epitopes recognised by the AAS are unknown.

The SDS-PAGE banding pattern obtained using the different MAbs and AASs, consisted of a doublet with a 43,000-44,000 band which corresponds to the neuraminidase treated class I heavy chains and is consistent with those of other mammals. Additionally, a 39,000-40,000 band was also observed. These smaller molecules, are associated with β_2m given their recognition by W6/32 and B1.1G6. Their sensitivity to neuraminidase, suggests that they are sialated and have traversed the trans-golgi network (Goochee and Monica,1990). Furthermore, their presence the detergent phase implies that they retain their hydrophobic region, indicating that they are membrane associated. Therefore, they are most likely generated by proteolysis of larger molecules by natural cellular events. Such events have been reported previously in both humans and cattle (Krangel,1986; Bensaid et al.,1988). The use of protease inhibitors largely reduces the presence of these molecules on SDS-PAGE with little effect on the complexity of 1D-IEF (figure 4.8a,b). Bovine β_2m (14000) was not observed because it lacks methionine residues (Groves and Greenberg,1977).

FIGURE 4.2
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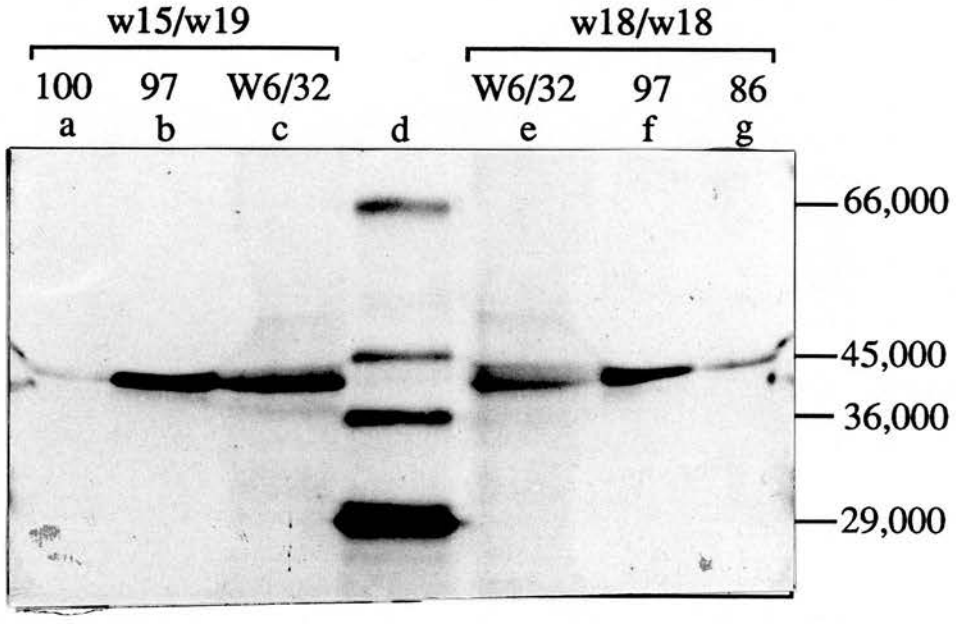
MOLECULAR MASS OF MOLECULES IMMUNOPRECIPITATED WITH AAS, ED86, ED97, ED100.

12% SDS-PAGE analysis of neuraminidase treated molecules immunoprecipitated from animals with the serological types w15/w19 and w18/w18 (in collaboration with Mr.R.Oliver).

Lanes a, b and c represent samples from an animal with serotypes w15/w19 immunoprecipitated with AAS ED100 (w19 specific), ED97 (w6 broad) and MAb W6/32 as control respectively. Lane e represents the molecular mass markers (values indicated to the right of the picture). Lanes f, g and h represent samples from an animal with serotypes w18/w18 immunoprecipitated with MAb W6/32 as control and AAS ED97 (w6 broad) and ED86 (w18 specific) respectively.


w15/w19
w18/w18
100
97
W6/32
W6/32
97
86
a
b
c
d
e
f
g
66,000
45,000
36,000
29,000

4.1.2. BoLA class I heterogeneity in 1D-IEF:

The 1D-IEF patterns obtained using any of the three MAbs, W6/32, B1.1G6 and IL-A88 were very complex. The results are shown in figure 4.3. It was observed that the patterns produced by MAbs W6/32 and B1.1G6 are very similar. However, the bands precipitated by B1.1G6 were generally less intense than those precipitated with W6/32. MAb IL-A88, on the other hand, showed the ability to precipitate an additional range of molecules with basic pI points not seen with W6/32 or B1.1G6, referred to as IL-A88$^+$ molecules (figure 4.3c and d). IL-A88, however, exhibited poor reproducibility in its ability to precipitate these molecules. The molecules shared in the patterns produced using the three MAbs are most likely to represent class I heavy chains that are associated with β_2m given that W6/32 requires β_2m for its reaction and B1.1G6 is directed against β_2m. To determine the nature of the IL-A88$^+$ molecules, a sequential immunoprecipitation method was used whereby W6/32 was used in the first round of immunoprecipitations (three cycles) and IL-A88 in the second. The resultant samples were analysed by 12% SDS-PAGE and 1D-IEF, the results are shown in figures 4.4a and 4.4b. It appeared that the molecular mass of the molecules, which constitute the difference between the two patterns (IL-A88$^+$), is approximately 39,000, 4,000-5,000 less than those recognised by W6/32. These molecules are possibly surface expressed molecules that have dissociated from β_2m at the cell surface due to the absence of, or ill association with, antigenic peptide (Rock et al.,1991), which would explain the inability of either W6/32 or B1.1G6 to recognise them. Such molecules are more sensitive to proteolytic digestion than β_2m associated molecules (Owen et al.,1980 and Ploegh et al.,1979), which could explain their

FIGURE 4.3
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1D-IEF PATTERNS FROM IMMUNOPRECIPITATIONS WITH MAbs W6/32, B1.1G6 AND IL-A88.

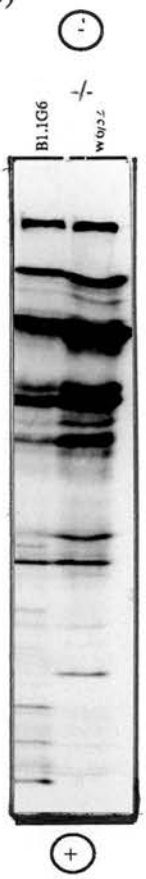
Figures 4.3a and b show a comparison between the 1D-IEF patterns obtained with MAbs W6/32 and B1.1G6. The animals used had the serological types w18/Ed99 (4.3a) and -/- (4.3b).

Figures 4.3c and d show a comparison between the 1D-IEF patterns obtained with MAbs W6/32 and IL-A88. The animals in this case had the serological types w14/w17 (4.3c) and w18/w18 (4.3d).

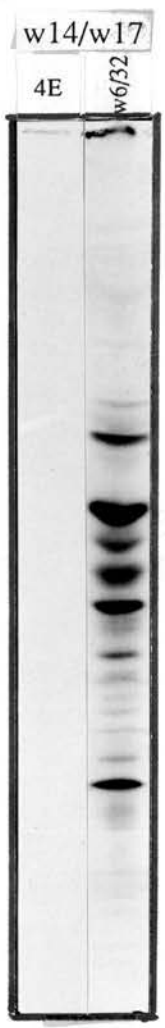
Figure 4.3e shows a comparison between an immunoprecipitation with MAb W6/32 and the negative control MAb 4E.

(4.3a)

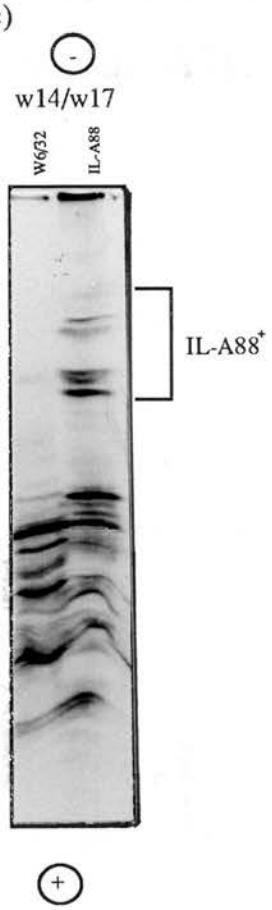
(4.3b)



(4.3e)



(4.3c)



(4.3d)

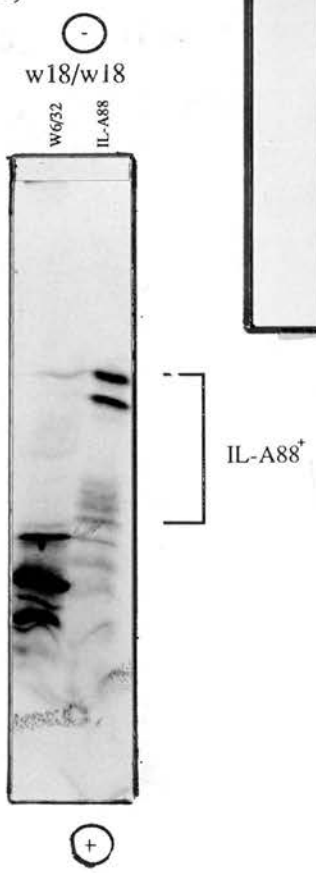


FIGURE 4.4
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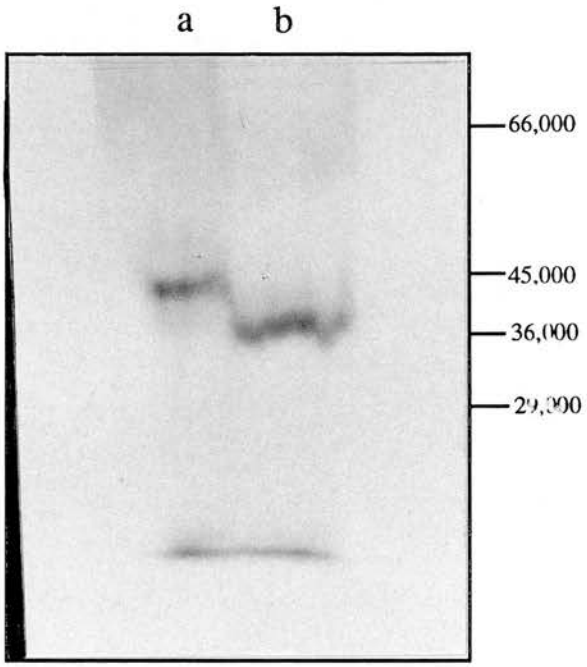
SDS-PAGE and 1D-IEF ANALYSIS OF SEQUENTIAL IMMUNOPRECIPITATES WITH W6/32 AND IL A88.

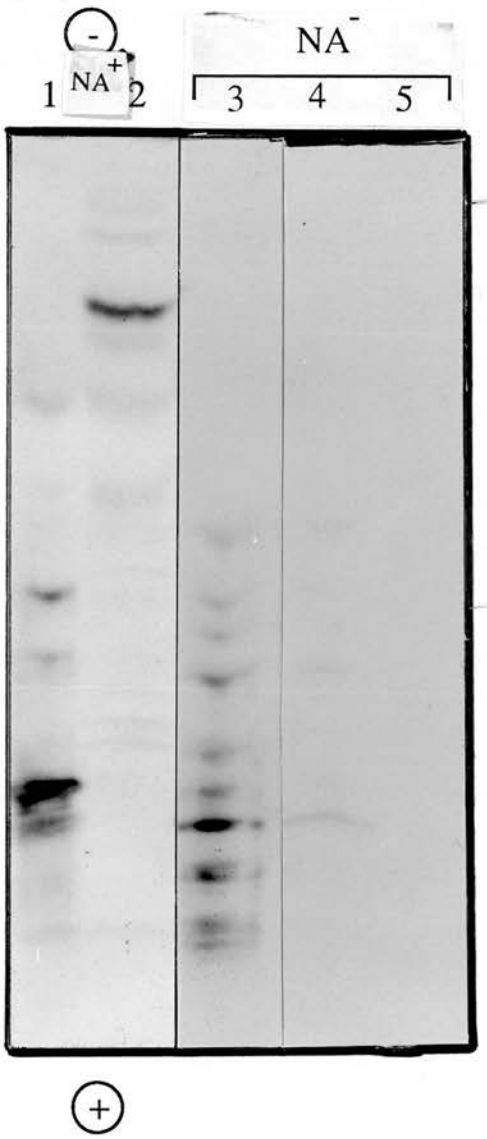
Figure 4.4a shows results from SDS-PAGE analysis, lane a, represents the samples after a first round of immunoprecipitations with the MAb W6/32. Lane b, represents the samples after the second round of immunoprecipitations with the MAb IL-A88. The molecular mass markers are indicated to the right of the figure.

Figure 4.4b represents the 1D-IEF patterns of the samples described in figure 4.4a, lane 1 is the sample immunoprecipitated with W6/32, lane 2 represents the IL A88$^+$ molecules. Lanes 3, 4 and 5 show the products of the, second, third and fourth cycles of immunoprecipitation with W6/32, respectively. Samples in lanes 3, 4 and 5 were not treated with neuraminidase.

(4.4a)



(4.4b)



lower molecular mass. The class I heavy chains which leave the ER have no absolute requirement for the association with β_2m. Such molecules become fully glycosylated and reach the cell surface, albeit at low levels (Hansen et al.,1988). Therefore, the IL-A88$^+$ molecules could arise by either dissociation from β_2m at the cell surface or by totally escaping this association during biosynthesis. Other explanations for the presence of IL-A88$^+$ molecules cannot be completely discounted. Polymorphism at the β_2m encoding locus could also be responsible for the inability of W6/32 and B1.1G6 to recognise these molecules, by having epitopes which are different to those required by these MAbs. Such polymorphism in β_2m has been reported in mice (Perarnau et al.,1990). Also, the IL-A88$^+$ molecules could simply lack the appropriate epitope required for recognition by W6/32. These molecules could also be non-MHC molecules that co-precipitate with MAb IL-A88.

Class I heavy chains that fail to maintain their association with β_2m are unstable and therefore thought to be functionally inactive (do not take part in antigen presentation) and are turned over quickly. They do, however, persist at the cell surface for at least one hour (Ortiz-Navarrete and Hammerling,1991; Rock et al.,1991). Their transient presence on the cell surface would explain their appearance in the detergent fraction, it would also explain the poor reproducibility of those basic patterns when IL-A88 is used. It was also suggested recently that the class I molecules expressed on the surfaces of β_2m-deficient cells have the ability to function as antigen presenting molecules (Glas et al.,1992).

The confirmation that the molecules detected by this method are actually found on the cell surface came from the observation that the 1D-IEF patterns obtained with

W6/32 after metabolic labelling were almost identical to the patterns obtained with W6/32 after surface labelling with ^{125}I. The differences were in faintly labelled molecules (two or three) with more basic pI points that are only seen clearly after iodination, they appear very faint or not at all after metabolic labelling. This differential labelling indicates that these molecules either lack or have very few methionine residues and that their levels of expression are much lower than the more acidic molecules in the pattern (R.Oliver, personal communication). Additionally, bovine β_2m (basic pI) could only be seen after iodination due to it's lack methionine residues (Groves and Greenberg,1977). It should be stressed, however, that the majority of the molecules in the patterns observed are identical regardless of the labelling method employed. For this reason metabolic labelling was carried out throughout the experiments described in this section.

4.2.POST-TRANSLATIONAL MODIFICATIONS AND CLASS I MHC CHARGE HETEROGENEITY:

Serological studies suggest the existence of a single highly polymorphic locus encoding BoLA class I molecules. This conclusion was made after the observation that the BoLA phenotype was usually determined by two allo-antisera with no evidence of recombination (Anon,1982; Bull et al.,1989; Bernoco et al.,1992). The use of 1D-IEF, however, reveals the presence of a complex banding pattern of BoLA class I molecules from each animal. This finding questioned the origin of these molecules. If the BoLA system had a single encoding locus then this heterogeneity in the IEF pattern would have to be the product of post-translational modifications. These include

glycosylation, phosphorylation and alternative splicing of pre-mRNA. This section reviews the results of experiments to determine whether the complex 1D-IEF pattern is indeed a result of differences in the post-translational modification of a small number of allelic products, two in the case of a single functional locus.

4.2.1. Glycosylation:

The oligosaccharide side chains of glycoproteins are assembled while the newly synthesised proteins are moving through the secretory pathway. Glycoproteins can carry carbohydrates attached to aspargine (N-linked glycans), and to serine and threonine (O-linked glycans). N-linked glycosylation starts with the assembly of a lipid-linked oligosaccharide chain followed by transfer en bloc onto the nascent protein. The subsequent steps are initiated in the ER and completed in the trans-golgi network (Brindle,1991).

MHC class I molecules are glycoproteins. HLA class I molecules have been reported to have a single available site for N-linked glycosylation at aspargine 86 (Parham et al.,1977; Ploegh et al.,1981). Direct chemical analysis of the carbohydrate moiety of the HLA molecules reveals them to have an approximate molecular mass of 3,000 (Parham et.al.,1977). Ploegh and co-workers (1981) reported that the deglycosylated HLA class I molecules analysed using SDS-PAGE exhibited a shift in molecular mass corresponding to 4,000. The differences in the two reports could be explained by the poor capacity of the carbohydrate moiety to bind SDS. The estimation of the molecular mass of glycoproteins using SDS-PAGE always results in

higher than normal estimates. Generally, however, these errors in estimation diminished with the decreasing porosity of the gels (Leach et al.,1980). The class I molecules of the murine H-2 system are also glycosylated at the same position. However depending on the class I heavy chain expressed, there could be up to two other oligosaccharide molecules present on mature H-2 molecules at positions 86, 176 and 256 (Kimball et al.,1981). N-linked glycosylation has been reported to have an effect on the conformation of the class I heavy chain and its subsequent assembly with β_2m (Neefjes and Ploegh,1988). Such conformational changes induced by glycosylation or the lack of it may alter the ability of MHC molecules to interact with $CD8^+$ T cells (Black et al.,1981; Boog et al.,1989 and Neefjes et al.,1990b).

To determine the effect of glycosylation on the complexity of the 1D-IEF patterns observed for bovine class I molecules, samples were treated with neuraminidase (NA) which removes all sialic acid residues, and endoglycosidase F/N-glycosidase F (EndoF) which removes all the sugar molecule, en bloc, from the amino acid backbone. Figures 4.5 and 4.6 show the reproducible results from the treated samples analysed by 12% SDS-PAGE and 1D-IEF respectively. The 12% percentage used for SDS-PAGE combines good separation of molecular masses between 20,000-60,000 while having a suitable level of porosity for the estimation of the molecular mass of the bovine class I molecules with little associated error. From figure 4.5, the molecular mass markers are indicated to the right of the picture. Lane a represents the sample after treatment with neuraminidase which caused a shift in the position of the molecules on the gel corresponding to a change of approximately 1,000-1,500 in molecular mass. From the fact that the molecular mass of sialic acid is 309, this

FIGURE 4.5
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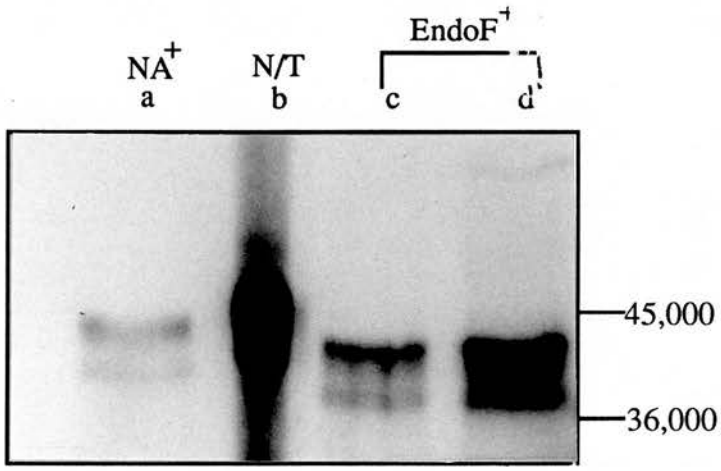
SDS-PAGE ANALYSIS OF GLYCOSYLATION OF BoLA CLASS I MOLECULES.

All samples were immunoprecipitated with W6/32. Lane a represents samples treated with neuraminidase (NA^+). Lane b represents the immunoprecipitated sample which received the same treatment as the other sample but without being treated with any enzyme (N/T). Lanes c and d are samples treated with 0.1 and 0.3U of EndoF ($EndoF^+$) respectively. Once again the distance between the heavy and light bands of the doublet is maintained. However, the whole of the doublet loses approximately 4000 from its apparent molecular mass. The molecular mass markers are indicated to the right of the picture.


NA+
a
N/T
b
EndoF+
c
d
45,000
36,000

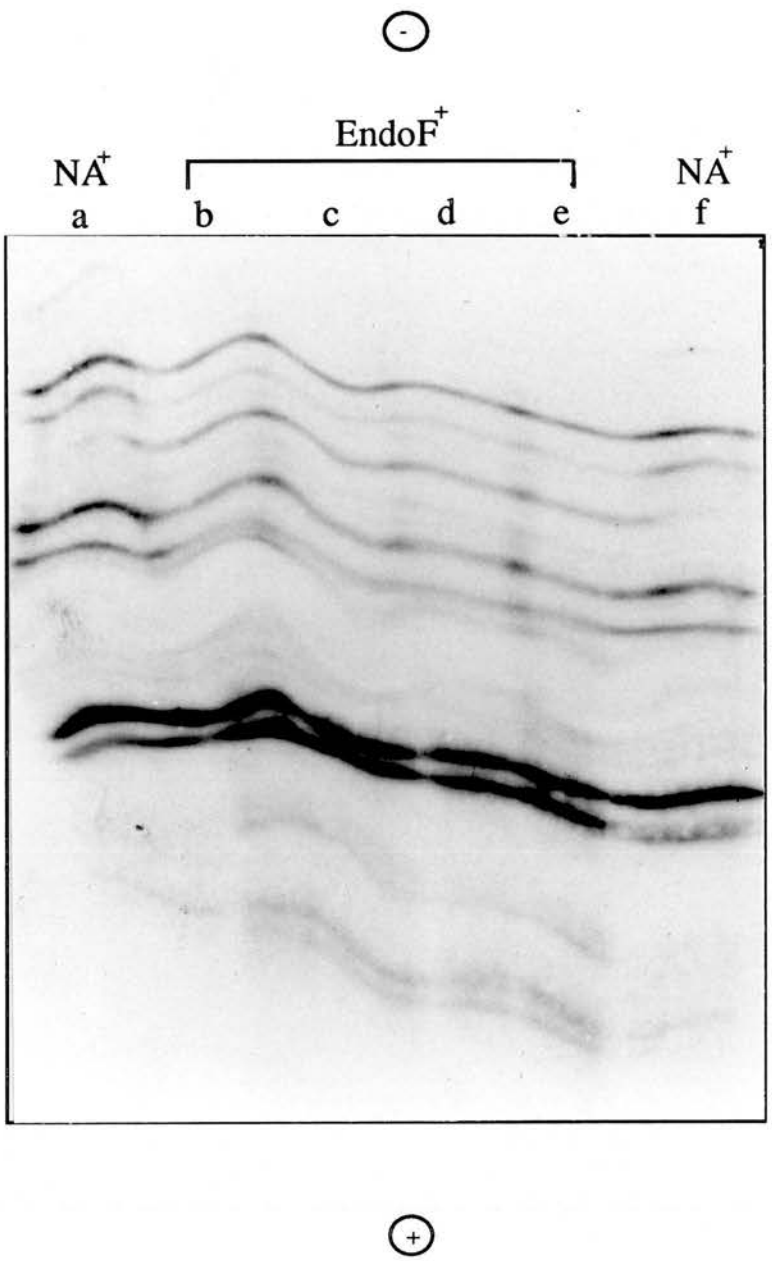
observation indicated that the sugar moiety on bovine class I molecules carries 3-4 residues. Due to the errors involved in the estimation of the molecular masses of glycoproteins using SDS-PAGE, the terminal sialic acid residues are likely to be 2-3 (as in humans) rather than the estimated 3-4 residues estimated here. Lane b represents the W6/32 immunoprecipitated sample with no enzymatic treatment. Treatment with endoglycosidase F/N-glycosidase F causes a shift that corresponded to a 4000 change in molecular mass (lanes c and d), which corresponds with the presence of a single N-glycosylated site on BoLA molecules. The doublet pattern described previously was maintained after both digestions. This indicates that these smaller molecules are in fact fully glycosylated and have traversed the golgi apparatus and further supports the suggestion that they are mature molecules that have undergone proteolysis as is described in section 4.1.2.. The results also indicate that the enzymatic digestion by NA and EndoF was complete.

Figure 4.6 shows the 1D-IEF patterns of the samples after treatment with NA and EndoF. Lanes a and f represent the samples after treatment with neuraminidase. Lanes b, c, d and e represent the samples incubated with a variety of EndoF concentrations. Treatment of the samples with neuraminidase led to a shift in the position of all the molecules present to a more basic position and to a reduction in the heterogeneity of the pattern (Fig. 4.4b). The samples treated with EndoF showed 1D-IEF patterns similar to those achieved by neuraminidase treatment alone, regardless of the larger shift in molecular mass observed with EndoF digestion (Fig. 4.5). These results indicated that removal of sialic acid whether direct (NA) or indirect (EndoF) results in a reduction in the charge heterogeneity of bovine 1D-IEF patterns.

FIGURE 4.6
Facing page

ANALYSIS OF THE GLYCOSYLATION OF BoLA CLASS I MOLECULES BY 1D-IEF.

All samples were immunoprecipitated with W6/32. Lanes a and f represents samples treated with neuraminidase (NA^+) in the normal way. Samples b, c, d and e were treated with 0.1U, 0.2U, 0.3U and 0.4U of EndoF ($EndoF^+$) respectively.


⊖
EndoF+
NA+
NA+
a
b
c
d
e
f
⊕

These results indicate that the complexity of the 1D-IEF pattern from the neuraminidase treated immunoprecipitations of class I molecules from the PBL of different animals could not be explained simply as differences in the extent of glycosylation of a small number of allelic products. The results also indicate that there is a single N-linked glycosylated site on bovine class I molecules and that the carbohydrate moiety is complex and could have up to four sialic acid residues. This is in agreement with results obtained from work with HLA molecules (Parham et al.,1977; Ploegh et al.,1981). The possible effect of proteolytic digestion resulting from impurities in the enzymes (NA and EndoF) was not addressed and although unlikely, their presence could be a source of error (see discussion).

The amino acid sequences available for BoLA class I molecules are also in agreement with the fact that only a single N-linked glycosylation site is available at position 86, as described in section 4.5 (Ennis et al.,1988; Brown et al.,1989 Bensaid et al.,1991).

4.2.2. Phosphorylation:

The phosphorylation of proteins involves the addition of a negatively charged phosphate group to serine and possibly tyrosine residues. It is thought that phosphorylation functions as one of the signals required for the internalisation of the protein (Capps et al.,1989).

HLA molecules have a single serine phosphorylation site located in the cytoplasmic tail of the molecules (Pober et al.,1978). Loube and co-workers (1983) reported that only a minor proportion of the total cellular class I molecules is actually

phosphorylated. This was augmented by a report by Eichholtz and co-workers (1992) where it was suggested that less than 1% of the class I molecules are phosphorylated at resting levels. The investigation of the available bovine class I sequences suggests that they also have a potential phosphorylation site in the cytoplasmic tail (Parham et al.,1988; Brown et al.,1989 and Bensaid et al.,1991).

The phosphorylation of a given amino acid backbone would give the protein a negative charge, hence, affecting its mobility in 1D-IEF. Therefore, the same class I allelic product could exhibit two different mobilities, or indeed more depending on the number of phosphorylated residues present. This differential phosphorylation of the same molecule could be responsible for the heterogeneity in the IEF patterns obtained from bovine cells. To investigate this possibility PBM were harvested in the usual manner. Samples containing 10^7 cells were incubated in methionine free or phosphate free culture medium (150mM NaCl, 5mM $MgCl_2$, 5mM KCl, 1.8mM Glucose, 2mM Glutamine and 10mM Tris-acetate pH 7.4; Pober et al.,1978) for 30 mins. and 90 mins. respectively. Subsequently, they were either labelled with ^{35}S methionine overnight at a concentration of 25uCi/ml or with carrier free $^{32}P_i$ at 80uCi/ml for three hours. The cells were then lysed in TX114 buffer containing phosphatase and protease inhibitors (0.5ml TX114, 0.5ml 1M $MgCl_2$, 10ml 0.5M Tris-Cl pH 7.4, 0.4mM EDTA, 10mM NaF, 10mM sodium phosphate, 0.4mM sodium vanadate, 1mM PMSF and 10mM Iodoacetamide). The class I molecules were immunoprecipitated with the monoclonal W6/32. All the samples were treated with neuraminidase. Samples were then used in SDS-PAGE and 1D-IEF analysis in the usual way. The results are shown in figures 4.7 and 4.8a,b.

FIGURE 4.7
Facing page

SDS-PAGE ANALYSIS OF ^{32}P LABELLED TOTAL CELL LYSATE.

Cells were metabolically labelled with $^{32}P_i$ for 3 hours, lysed in sample buffer, boiled and applied to a 12% SDS-PAGE gel. A number of Phosphoproteins are observed (arrows).

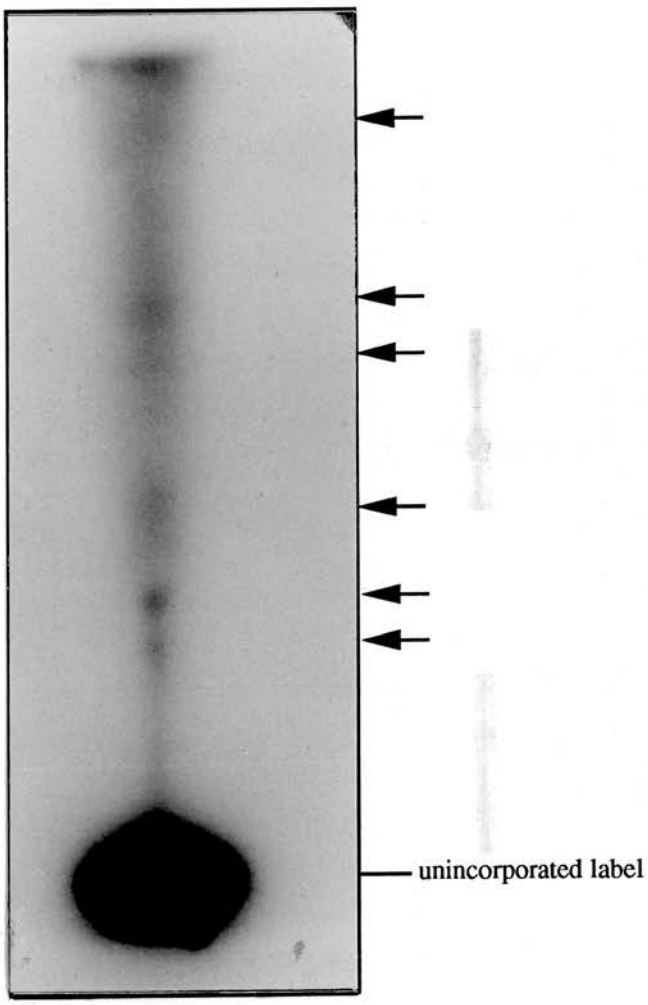

unincorporated label

FIGURE 4.8
Facing page

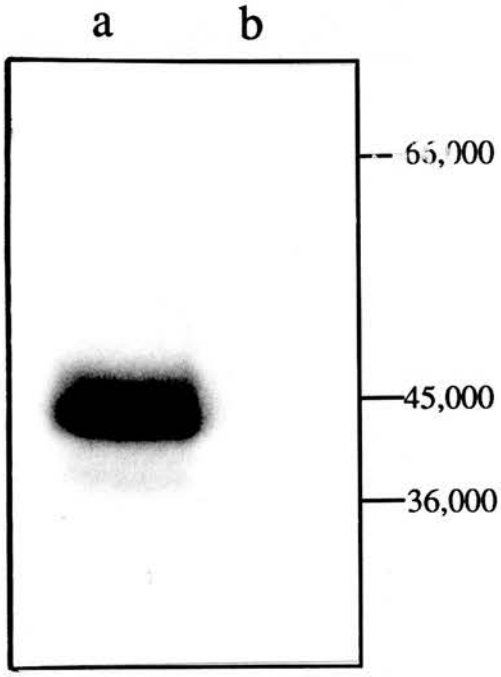
SDS-PAGE AND 1D-IEF ANALYSIS OF W6/32 IMMUNOPRECIPITATED CLASS I MOLECULES FROM $^{32}P_i$ LABELLED CELLS.

Figure 4.8a shows W6/32 immunoprecipitated samples were analysed on 12% SDS-PAGE gels and compared to ^{35}S labelled molecules immunoprecipitated with the same MAb.

Lane a represents the ^{35}S labelled sample. Lane b represents the ^{32}P labelled sample. The molecular mass markers are indicated to right of the figure. Protease and Phosphatase inhibitors were included in the lysis buffer.

Figure 4.8b shows the results of 1D-IEF analysis of the samples described in 4.8a. The sample sequence was as above.

(4.8a)



(4.8b)

Figure 4.7 shows the X-ray (2 hours at -70°C) of complete cell lysate from cells labelled with $^{32}P_i$ for 3 hours. The sample was run on a 12% SDS-PAGE gel. A number of phosphorylated protein species could be identified, and unincorporated label was also detected.

SDS-PAGE analysis of detergent extracted W6/32 immunoprecipitated molecules from cells labelled with either ^{35}S or $^{32}P_i$ is illustrated in figures 4.8a. Only ^{35}S labelled proteins were detected. These results indicate that the surface bound bovine class I molecules are not phosphorylated. This finding correlates with the findings of Loube and co-workers (1983) and is also in agreement with the notion that phosphorylation is a signal for protein internalisation. Figure 4.8b shows the same samples after 1D-IEF analysis, once again no phosphorylated molecules were observed.

In another approach to address the same problem, the electrophoretic mobility of ^{35}S methionine labelled, neuraminidase treated class I molecules was tested before and after treatment with the enzyme potato acid phosphatase. The pK_a of the phosphate group in serine phosphate is 5.91 (Dawson et al.,1986). At pH 5.9 half of the phosphate will be electrophoretically silent, at pH 5.3 80% will be un-ionised. Because most of the class I MHC molecules that have an intact sialic acid complement exhibit more acidic isoelectric points (pIs) (Loube et al.,1983), it was thought that treating with neuraminidase first would shift the class I molecules to more basic positions, therefore giving phosphorylated molecules the chance to express differences in their mobilities on 1D-IEF gels. The results are given in figures 4.9 and 4.10 for SDS-PAGE and 1D-IEF respectively.

Figure 4.9 shows the SDS-PAGE analysis of samples immunoprecipitated with W6/32 without subsequent enzyme treatment, samples treated with neuraminidase only and samples treated with neuraminidase followed by potato acid phosphatase. Previous studies involving HLA class I molecules have indicated that the phosphorylated class I molecules from the HLA and H-2 systems have a molecular mass that is approximately 1000 more than the non-phosphorylated molecules (Loube et al.,1983; Capps and Zuniga,1990). Figure 4.9 shows that no such differences in mobility were observed with detergent solubilised bovine class I molecules which exhibited similar mobilities whether treated with neuraminidase alone or neuraminidase followed by potato acid phosphatase, and there was no net difference in migration that could be attributed to differences in phosphorylation. These findings were augmented by the 1D-IEF analysis of these samples shown in figure 4.10. From this figure it could seen that the bovine class I molecules had identical 1D-IEF patterns before and after treatment with potato acid phosphatase. These findings further support the results described above and indicate that the surface expressed bovine class I molecules are not phosphorylated and that the heterogeneity in the IEF patterns observed for different animals could not be attributed to differences in the phosphorylation of a small number of polypeptides.

The activity of the potato acid phosphatase used in these studies was assessed by testing its ability to dephosphorylate whole milk proteins from transgenic mice (fig. 4.11). Three lines of mice P.C./40.1, P.C./46 and P.C./66.8 containing an insert for the ovine protein β-lactoglobulin (BLG) were used. This particular insert constituted nine amino acids and had four potential phosphorylation sites carried on an external loop

FIGURE 4.9
Facing page

ANALYSIS OF THE PHOSPHORYLATION OF BoLA CLASS I MOLECULES BY SDS-PAGE.

All samples were immunoprecipitated with W6/32 and Neuraminidase treated. Lanes a, b and c represent samples treated with neuraminidase and with 0.15U (3hrs), 0.25U (3hrs) and 0.15U (overnight) of potato acid phosphatase (PAP^+/NA^+) respectively for 3hrs. Lane d represents a sample treated with nuraminidase only (NA^+). Lane e represents a sample that received the same treatment as the rest of the samples but without any enzyme (N/T). The molecular mass markers are indicated to the right of the figure. No shift in molecular mass that could be attributed to phosphorylation was observed.

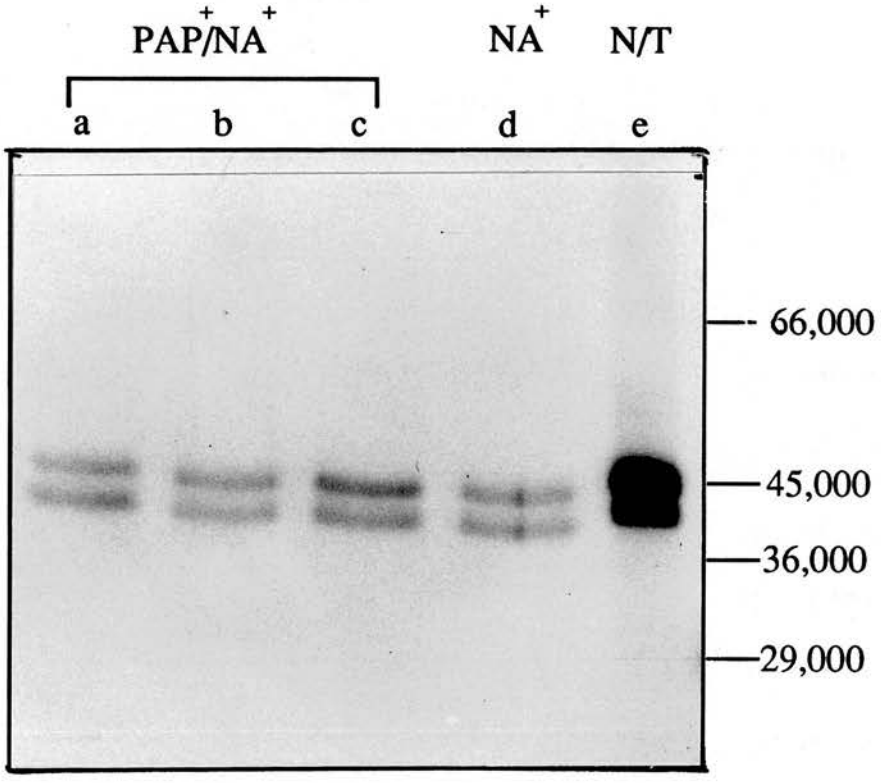

PAP/NA+ +
NA+
N/T
a
b
c
d
e
66,000
45,000
36,000
29,000

FIGURE 4.10
Facing page

ANALYSIS OF THE PHOSPHORYLATION OF BoLA CLASS I MOLECULES BY 1D-IEF.

All samples were immunoprecipitated with W6/32. Lane a represents a samples treated with neuraminidase (NA^+) only. Lane b, represents a sample treated with neuraminidase and 0.15U (6hrs) of potato acid phosphatase (NA^+/PAP^+). No reduction in the charge heterogeneity was observed and there was no change in pIs.


⊖
NA+
a
NA+/PAP+
b
⊕

of the protein starting at position 85. The milk was defatted and samples from each line were either treated with potato acid phosphatase or left untreated. In the treated samples, 0.5U of the enzyme was added and the digestion carried out for 5 hours (0.25U for 3 hours is sufficient). The results are shown in Figure 4.11. A shift in the position of α and β casein as well as BLG was observed after potato acid phosphatase treatment. The extent of the shift in the case of BLG was not large enough to bring the genetically engineered molecules back to a position similar to those migrated to by normal purified BLG, even after taking into account the added mass of the insertion. These results indicated that the genetically engineered phosphorylated form of BLG has a slightly different structure from that of the natural protein, and that an extended form (maintained by SDS) of the genetically engineered structure will also be different from the extended form of the natural protein and could be responsible for the abnormal migration observed for the dephosphorylated protein. The results also indicate that the enzyme is active and that the phosphorylation of proteins retards their migration, at least in part, in SDS-PAGE systems.

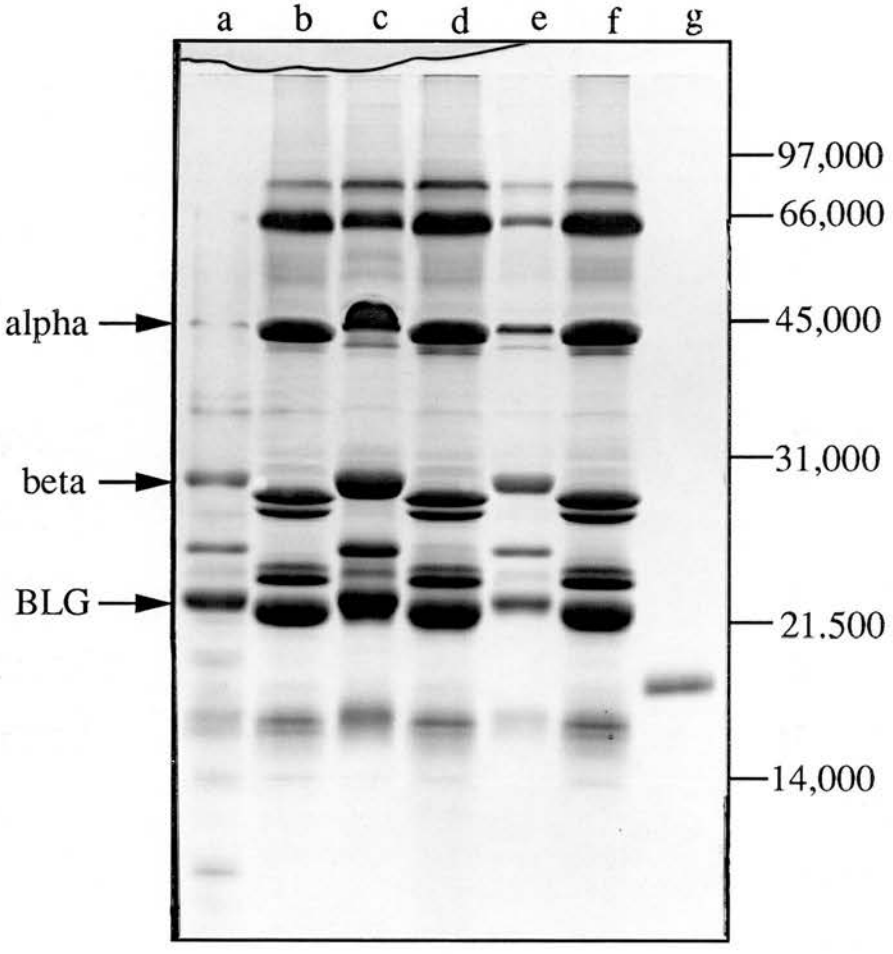
The work involving the transgenics was carried out as part of a collaborative effort with Drs.E.Hitchin, M.McClenaghan and Mr.K.Dobbie from the department of Molecular Genetics, Roslin Institute, Roslin, Midlothian, Scotland.

FIGURE 4.11
Facing page

ANALYSIS OF POTATO ACID PHOSPHATASE ACTIVITY BY 15% SDS-PAGE USING MOUSE WHOLE MILK PROTEIN.

Mouse whole milk protein was used to test the activity of the purchased potato acid phosphatase. The positions of the molecular weight markers are indicated to the right of the picture and only the relevant part of the gel is shown. Lanes a and b represent potato acid phosphatase treated and untreated samples respectively, taken from the transgenic mouse line P.C./40.1. Lanes c and d represent the treated and untreated samples, respectively, taken from the trasgenic mouse line P.C./46. Lanes e and f represent treated and untreated samples, respectively, taken from the transgenic mouse line P.C./66.8, lane g represents purified normal BLG.

The amount of potato acid phosphatase used with the treated samples was 0.5U (5hrs), however the same result could be achieved using only 0.25U of the enzyme (gel not shown). The arrows to the left indicate the position of the phophorylated forms of α *casein,* β *caseins and BLG.*


a
b
c
d
e
f
g
97,000
66,000
45,000
31,000
21.500
14,000
alpha
beta
BLG

4.2.3. Alternative splicing of pre-mRNA:

Alternative splicing in most cases is a mechanism that gives rise to protein isoforms that share extensive regions of identity, and varying only in specific domains, thus allowing for the fine modulation of protein function (Smith et al.,1989).

This mechanism involves the splicing of pre-mRNA at different sites and leads to different mRNA products from a single gene. Pre-mRNA observed in the class I system involves three basic forms of alternative splicing. In all three of these forms a segment canonically used as an exon is alternatively used as an intron, either completely (form A) or partially (form B). Additionally, a segment canonically used as a portion of an intron may be used alternatively as a portion of an exon (form C). All three types of alternative splices have been observed (Lew et al.,1987; Vogel et al.,1989 and Grossberger et al.,1990). Alternative splicing could occur in almost every exon. The products of alternative splicing at the 5' end of the pre-mRNA, for example, could increase the repertoire of antigenic peptides presented to the immune system. This form of alternative splicing has been rarely reported, and even when the mRNA was found no corresponding protein was found (Transy et al.,1984; Lalanne et al.,1985). The most abundant form of alternative splicing, occurs at the 3' end of the pre-mRNA. In all these reports the resultant class I molecules are either longer or shorter than the wild type protein (Archibald et al.,1986; Lew et al.,1986; McLuskey et al.,1986; Rogers et al.,1986; Vogel et al.,1986; Grossberger et al.,1990; Ishitani et al.,1992 and Ellis et al.,1993). It was also observed that the ratio of wild type to alternative splice form, whether it results in a shorter or longer molecule, was approximately 10:1. Therefore suggesting, that although alternative splicing is a viable

way of generating variability, it is rare in nature (Lew et al.,1987; Vogel et al.,1989).

The reasons behind alternative splicing are not fully understood. In the MHC system, isoforms of a certain class I molecule generated by this mechanism are unlikely to be the products of splicing errors because of their high rate of occurrence (5-10%) (Lalanne et al.,1985). Due to the fact that most alternative splice events occur at the 3' end of the pre-mRNA, it could reflect a certain function for the cytoplasmic domain of the class I molecules (Ellis et al.,1990). The form of alternative splicing which is most likely to have such functional importance, is the deletion of exon 7 which encodes the portion of the cytoplasmic domain accommodating the phosphorylation site (Mcluskey et al.,1986; Ellis et al.,1993). The exact role of the phosphorylation of class I molecules is unknown, however, it is thought that it could play a role in receptor signalling. The apparent physical interaction of MHC class I molecules with insulin receptors and other hormones (Fehlmann et al.,1985; Phillips et al.,1986; Edidin,1986), may implicate them in the transduction of hormone receptor-mediated signalling. It has been reported that this mechanism could be responsible for the generation of soluble forms of the class I molecules. The biological function of such molecules is yet to be determined (Lalanne et al.,1985; Krangel,1986 and Cianetti et al.,1989). Additionally, structural variation within the intracellular sequences of class I molecules may lead to different processing pathways (Zuniga and Hood,1986).

Alternative splicing could, therefore, be responsible for the generation of the observed heterogeneity in the BoLA 1D-IEF pattern. From the above, pre-mRNA alternative splices, if expressed, are most likely to give rise to protein products that differ in their sizes. Therefore, it was thought that such differences could be picked

up by two-dimensional (2D) SDS-PAGE analysis.

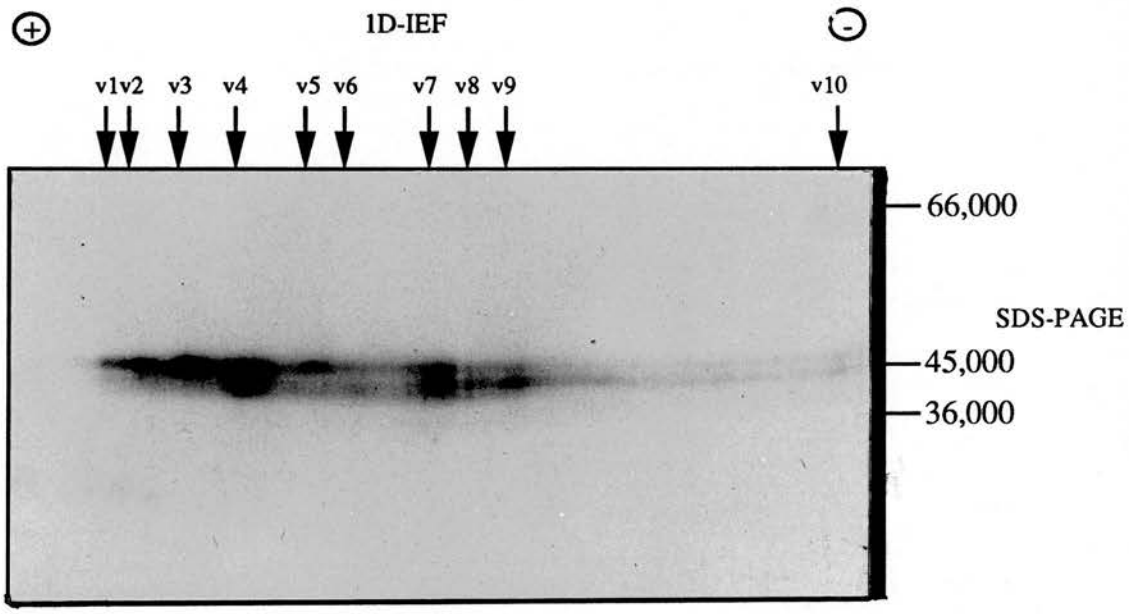
The results are given in figure 4.12 which shows the two dimensional pattern obtained from immunoprecipitations from lymphocytes exhibiting the serological type w14/w17. Up to 10 charge variants (v1-v10), were observed, only six of which (v4, v6, v7, v8, v9 and v10) showed doublet formations on the second dimension. The remainder of the molecules only exhibited the heavier of the two variants (44,000). These results suggest that if alternative splicing is responsible for the generation of the observed heterogeneity it must result in molecules that are identical in molecular mass, such a mode of alternative splicing has never been reported previously. This, in addition to the fact that the products of alternative splicing are usually expressed at around 10% of the amount of wild type protein, lead us to conclude that the observed heterogeneity, in the 1D-IEF patterns could not be generated by alternative splicing, although its occurrence could not be discounted completely.

The 2D patterns also suggested that a number of charge variants split into two species with differing sizes, once analysed on the SDS-PAGE second dimension. The presence of more than one doublet in the second dimension at pI positions identical to those of the heavier molecules argues against the possibility of the smaller molecules being totally different proteins that just happened to migrate to the same isoelectric point. It is more likely that these smaller molecules are actually identical to the larger variant but had been modified, probably by proteolytic enzymes before or during lysis. The most likely part of the molecule to be digested is the cytoplasmic tail. It is also apparent that the loss in molecular mass of approximately 4,000 from

FIGURE 4.12
Facing page

2D SDS-PAGE ANALYSIS OF BoLA CLASS I MOLECULES FROM LYMPHOCYTES WITH THE w14/w17.

The immunoprecipitation form an animal with the serotype w14/w17 was carried out using W6/32 and the sample was treated with neuraminidase. The first dimension is 1D-IEF, the second dimension is 12% SDS-PAGE. Ten charge variants (v1-v10) are observed all of which have the same molecular mass (44,000). The molecular mass markers are indicated to the right of the figure.


⊕
1D-IEF
⊖
v1v2
v3
v4
v5
v6
v7
v8
v9
v10
66,000
SDS-PAGE
45,000
36,000

the amino acid backbone did not affect the net charge of these molecules indicating that they have an equal distribution of positively and negatively charged residues over their whole lengths, and the loss of any part does not affect the net charge of the molecule.

4.2.4. Heterogeneity in the amino acid backbone:

The experiments so far indicate that the complex banding patterns observed with 1D-IEF from samples obtained from immunoprecipitations of molecules found in detergent extracts of cell lysates, are not due to differential glycosylation events nor to differential phosphorylation events. Furthermore, results involving 2D analysis of these immunoprecipitates revealed that the heterogeneity observed could not be solely attributed to alternative splicing, although this mechanism could not be discounted completely.

The observed heterogeneity in IEF pattern could, however, be due to the expression of different allelic products from different class I loci. To investigate this possibility, peptide mapping was used with a slight modification to the original method reported by Cleveland and co-workers (1977). Here the partial digestion of 1D-IEF separated bovine class I molecules was carried out on the second dimension (15% SDS-PAGE). The endoproteinase Glu-C (V8) from Staphylococcus auerus was used in performing the proteolytic digestion at the interface between the stack and run gels. The patterns of peptides generated using this method are characteristic of the protein substrate and the proteolytic enzyme and are highly reproducible.

Figure 4.13 shows the pattern obtained from lymphocytes with the specificity w10/w11 with up to eight charge variants (v1-v8). The partial digestion patterns of all were different from each other. The intensity of labelling of the different molecules is different, this could depend on the number of methionine residues in each molecule. It could, on the other hand, depend on the affinity of W6/32 to the different molecules. From figure 4.13, molecules v1, v3, v5 and v6 all show complete digestion of the parent molecules. v1 and v6 show a single digestion product each. v2, v3, v4 and v5 each exhibited a digestion pattern with up to four peptides. v7 shows up to eight digestion peptides and v8 exhibits three such peptides.

Figure 4.14 shows the pattern obtained from lymphocytes with the serological type w14/w17. Seven molecules are seen in the pattern having a molecular mass of approximately 44000. Four of these molecules, v1, v2, v3 and v5, gave a digestion pattern with V8 protease. No detectable pattern was observed for v4, v6 and v7. v1 gave a pattern with up to four digestion products, v2 gave 2 digestion products, v3 and v5 both gave 6 observed digestion products respectively.

The observation that some molecules did not give rise to any digestion products could be given two possible explanations. the first is that these molecules do not have any digestion sites available for V8 protease. The second is that their digestion products simply do not contain any methionine residues, making them impossible to visualise using this method.

FIGURE 4.13
Facing page

PEPTIDE MAP OF BoLA CLASS I MOLECULES FROM LYMPHOCYTES WITH A w10/w11 SEROLOGICAL TYPE.

All molecules were immunoprecipitated with W6/32 and treated with neuraminidase. The first dimension is 1D-IEF. The second dimension is 15% SDS-PAGE. Digestion with V8 protease was performed for 30mins. in the interface between the stack and separation gels. The low molecular mass markers are shown to the right of the picture.

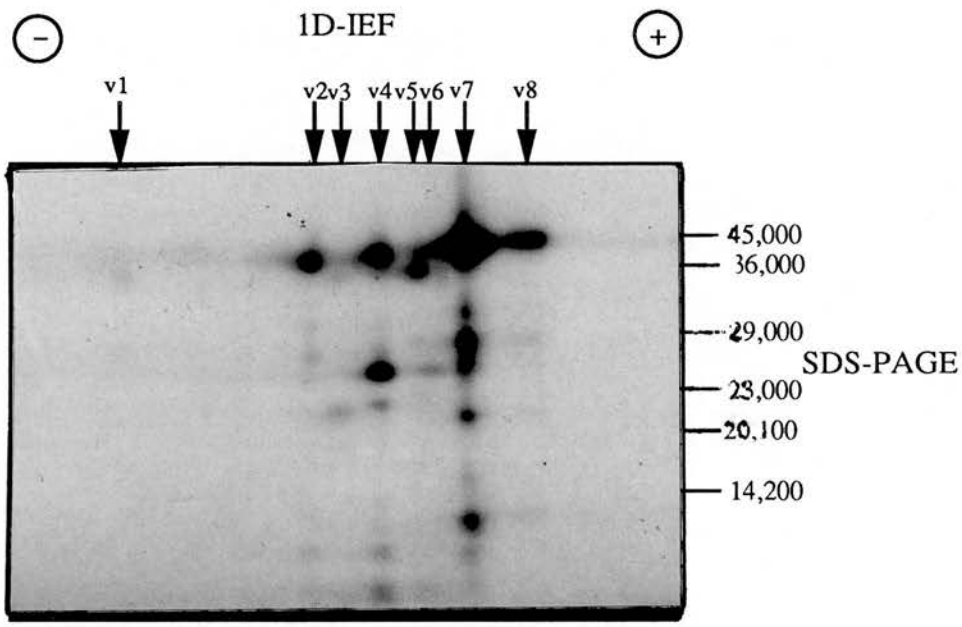

1D-IEF
v1
v2v3
v4
v5
v6
v7
v8
45,000
36,000
29,000
SDS-PAGE
23,000
20,100
14,200

FIGURE 4.14
Facing page

PEPTIDE MAP FROM BoLA CLASS I MOLECULES FROM LYMPHOCYTES WITH THE SEROLOGICAL TYPE w14/w17.

All molecules were immunoprecipitated with W6/32 and treated with neuraminidase.

The running conditions are as described in figure 4.13.

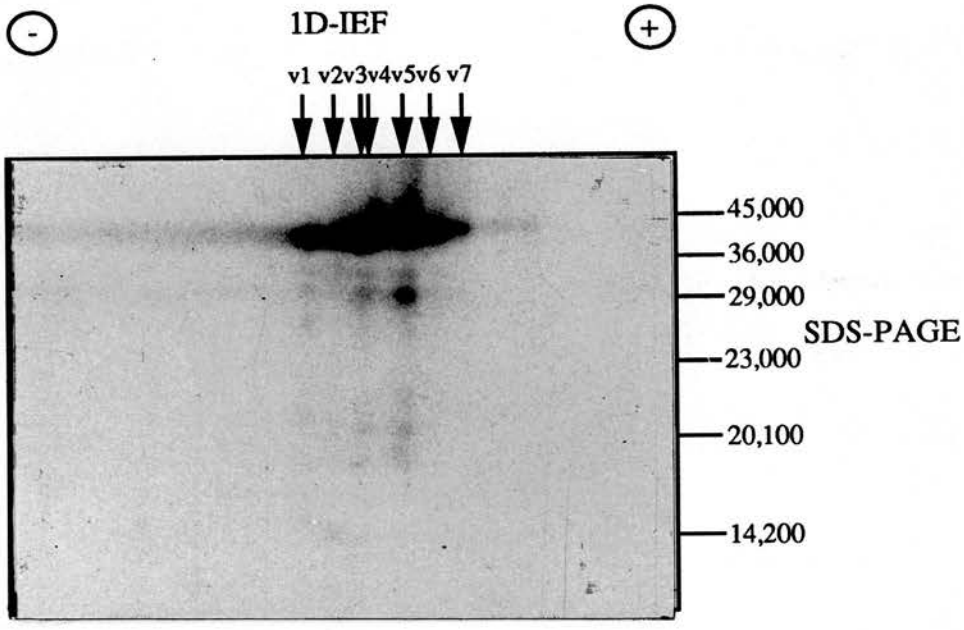

1D-IEF
-
+
v1 v2v3v4v5v6 v7
45,000
36,000
29,000
SDS-PAGE
23,000
20,100
14,200

Figure 4.15 shows the pattern obtained from lymphocytes exhibiting the serological specificity w10/w17. In this case up to five parent molecules were observed (v1-v5), all of which exhibited a defined digestion pattern. v1 had a single digestion pattern. v2 exhibited a digestion pattern with 4 digestion products, v3 and v4 gave identical patterns of 6 digestion products each. v5 gave 2 digestion products.

From the results above the following could be concluded: The v1 molecules in both animals w10/w11 and w10/w17 (Figs. 4.13 and 4.15, respectively) have identical digestion patterns and similar pIs on the first dimension. Joosten and co-workers (1992) reported that a molecule immunoprecipitated with the MAb IL-A31 raised against a polymorphic determinant on a BoLA molecule from the w10 serotype, gives a similar digestion pattern and migrates to a similar position on 1D-IEF as the molecule v1 seen in this experiment (Joosten and co-workers use the same 1D-IEF system as the one used here). The results described here suggest that v1 (Figs. 4.13 and 4.15) and the IL-A31 molecule are identical. v2 from animal w10/w11 exhibits a similar digestion patterns to v2 from animal w10/w17. Due to the fact that the w10 haplotype is shared between those two animals, it is likely that the v1 molecules are identical in both animals. Furthermore, the molecules v3 and v4 from animal w10/w17 (fig. 4.15) appear to be identical to molecules v3 and v5 from animal w14/w17 (fig. 4.14), and by the same argument above these molecules could be identical. Molecule v5 from the animal w10/w17 does not share its pattern with any of the molecules observed with the other animals.

We conclude that each BoLA serological specificity defines a number of different molecules that are inherited en bloc. Furthermore, this means that the

FIGURE 4.15
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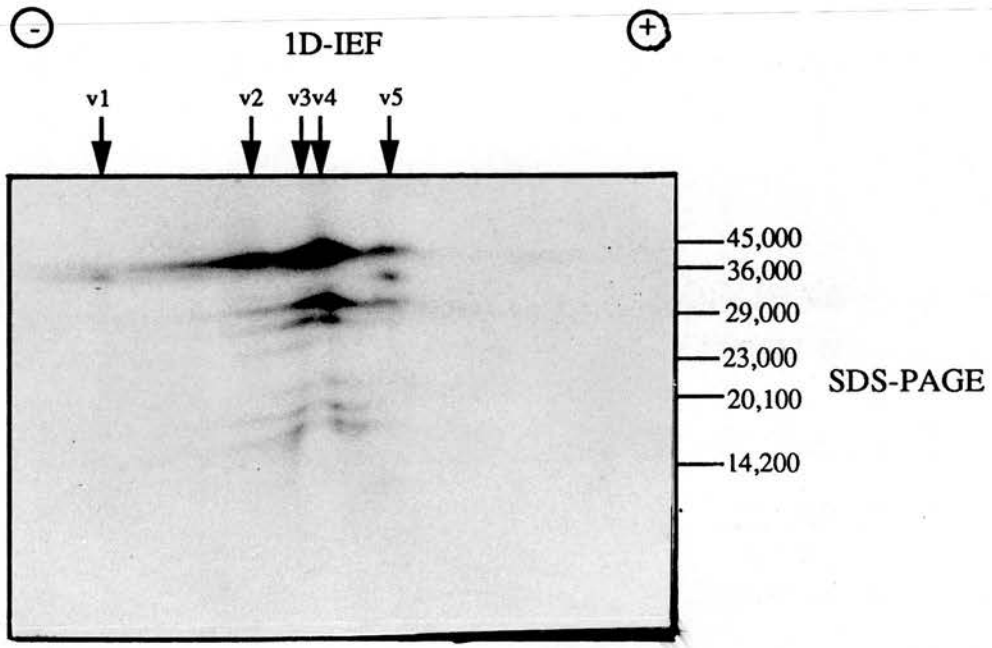
PEPTIDE MAP OF BoLA CLASS I MOLECULES FROM LYMPHOCYTES WITH THE SEROLOGICAL TYPE w10/w17.

All molecules were immunoprecipitated with W6/32 and treated with neuraminidase. The experimental condition were as described in figure 4.13. In this case the digestion pattern exhibited some similarities with the pattern obtained from the w10/w11 lymphocytes (figure 4.13).


-
1D-IEF
+
v1
v2
v3v4
v5
45,000
36,000
29,000
23,000
20,100
14,200
SDS-PAGE

allocation of the serological BoLA specificities to a single locus, BoLA-A, is false.

It must be noted at this point that MHC molecules from different species have very similar primary sequences. For example, the bovine sequence BL-6 shows up to 78% homology to HLA-A,B and C, and that BL-7 shows up to 82% homology with the consensus human sequence. The class I sequences from the same species have a much higher degree of homology (Ennis et al.,1988). Therefore, even products of different loci from the same species could have identical digestion patterns. Hence, it is difficult to allocate different molecules to different alleles and different loci using this technique. The only possible way of identifying individual allelic products of an individual locus with any confidence, is to obtain DNA sequence data. The above results, however, when taken with the combined results described in previous sections show that the different charge variants seen from the immunoprecipitates of class I molecules from individual animals did not arise from differential post-translational modification events. They also show that these variants have considerable differences in their primary sequences. The number of these variants indicates the expression of at least three class I loci on the surfaces of bovine PBL.

The use of comparative peptide mapping, using two or more proteases with contrasting specificities, could be most effective in obtaining a better picture as to the identity of individual molecules and remove any comformational artifacts that could obscure the specific sites of a given protease. Proteases such as V8, elastase, chymotrypsin, papain are only a few of the choices available for such an undertaking.

4.3. FURTHER EVIDENCE FOR THE EXPRESSION OF MORE THAN ONE CLASS I LOCUS:

The use of 1D-IEF produced differing multiple band patterns with samples from different animals. There is general agreement, relying on serological studies only that the class I molecules detected are the products of a single highly polymorphic locus "BoLA-A" (Bull et al.,1989 and Bernoco et al.,1991). The assignment of serological specificities to one locus was based on the observation that the MHC type of individual animals is usually defined by two AAS with no evidence of recombination (Anon,1982). This sharply contradicts the highly complex patterns obtained from 1D-IEF. It was therefore essential to investigate and dissect these banding patterns in order to better our understanding of their origins. To do so it was important to be able to allocate different bands in a 1D-IEF pattern to different serological specificites. Two approaches were used to this end. The first depended upon the visual comparison of various 1D-IEF patterns from the immunoprecipitation of the class I molecules from the PBLs of different animals using MAb W6/32. This MAb was chosen because it reacts with class I heavy chains only if associated with β_2m. w6/32 was preferred to B1.1G6 because it exhibited a stronger reaction to BoLA class I molecules. Further, W6/32 was preferred over IL-A88, because the latter recognises a linear epitope on class I heavy chains, therefore, has the potential of precipitating immature molecules (not associated with β_2m). The results from the visual comparison of the 1D-IEF patterns of different animals are given in figures 4.16, 4.17 and 4.18. The allocation of the bands to various serological specificities is given in the schematic representations accompanying each figure. In each case when

a band is allocated to a certain specificity then it will carry the number of that specificity in the schematic figure. Where a band could not be allocated to a certain specificity it will be marked "?" on the schematic figure. The sign (-) denotes an unknown serological specificity.

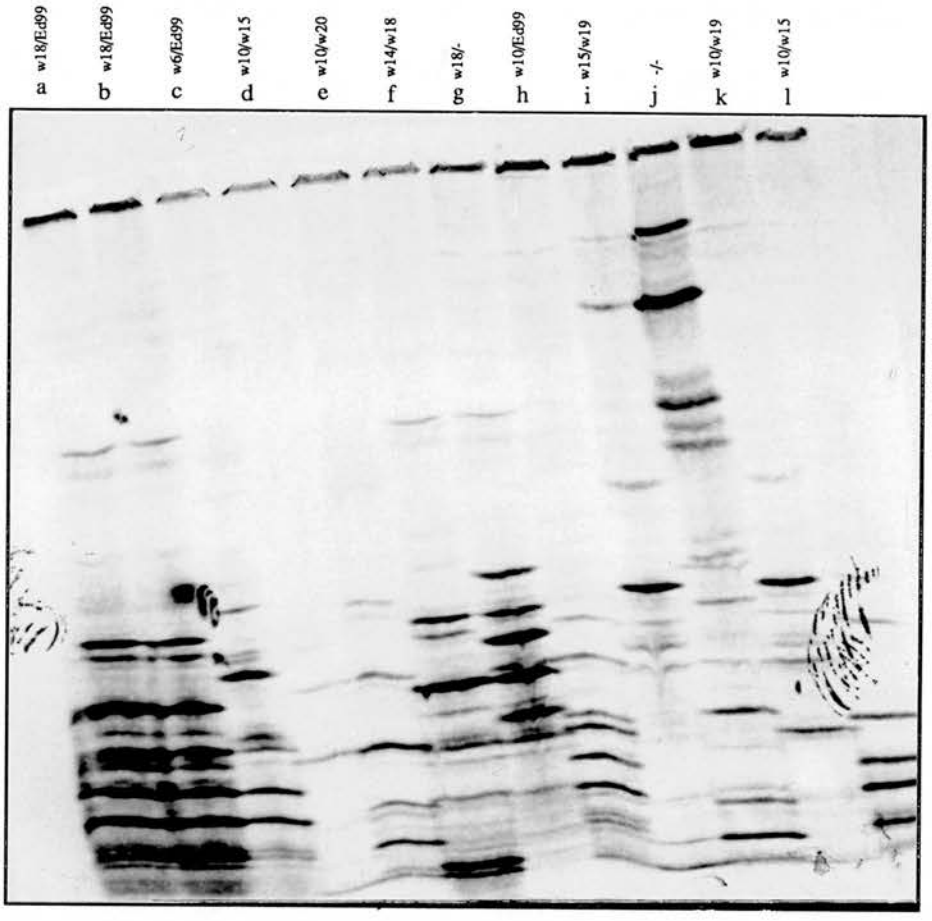
Figure 4.16 shows the IEF patterns of 12 different animals (11 British Friesians and one longhorn) produced using W6/32. The serological specificities represented were: w6, w10, w14, w15, w18, w19, w20 and ED99. Different serological specificities were allocated a different number of bands depending on the reproducibility of a certain pattern with a given serotype. From figure 4.16, specificities w14, w15, w20 coincide with the appearance of three acidic IEF bands. The serological specificities w6, ED99 and w19 are consistent with a pattern of 4-5 IEF bands. The ED99 bands appear to be the more acidic than those of w6 or w19, which have higher pI points. Seven IEF bands appeared to correspond to the serological specificity w18. The pI range of these bands varied widely as seen in figure 4.16. The specificity w10 showed a variation both in the number of bands observed and their pI point among different animals. The pattern in lane j of figure 4.16 was from the PBL of a Longhorn animal. This animal had no known serological type (-/-) and showed an IEF pattern that spanned the entire pH range used in our gels, with the most basic bands being more intense than the more acidic ones. The opposite of this was seen with the rest of the animals with the more acidic bands being more intense than those with higher pIs. Serological differences between breeds have been reported previously (Kemp et al.,1988). Manifestations of these differences are expected on 1D-IEF.

FIGURE 4.16
Facing page

DETERMINATION OF THE IEF BANDING PATTERNS FOR SEROTYPES: w6, w10, w14, w15, w18, w19, w20 and ED99 by visual comparison.

The class I molecules were precipitated using MAb W6/32. The serological types were as follows: Lanes a and b, w18/ED99, Lane c, w6/ED99, lane d, w10/w15, Lane e, w10/w20, Lane f, w14/w18, Lane g, w18/-, Lane h, w10/ED99, Lane i, w15/w19, Lane j, is a Longhorn animal with unknown serotypes, Lane k, w10/w19 and Lane l, w10/w15.

The bands corresponding to different specificities are indicated in the schematic representation. Bands are allocated to various specificities depending on the reproducibility of a particular banding pattern with a particular serotype. Bands that could not be allocated to any serological specificity are marked "?". The banding patterns showed consistent results with most specificities. Only animals with the w10 specificity showed inconsistencies in their banding pattern.


a w18/Ed99
b w18/Ed99
c w6/Ed99
d w10/w15
e w10/w20
f w14/w18
g w18/-
h w10/Ed99
i w15/w19
j -/-
k w10/w19
l w10/w15

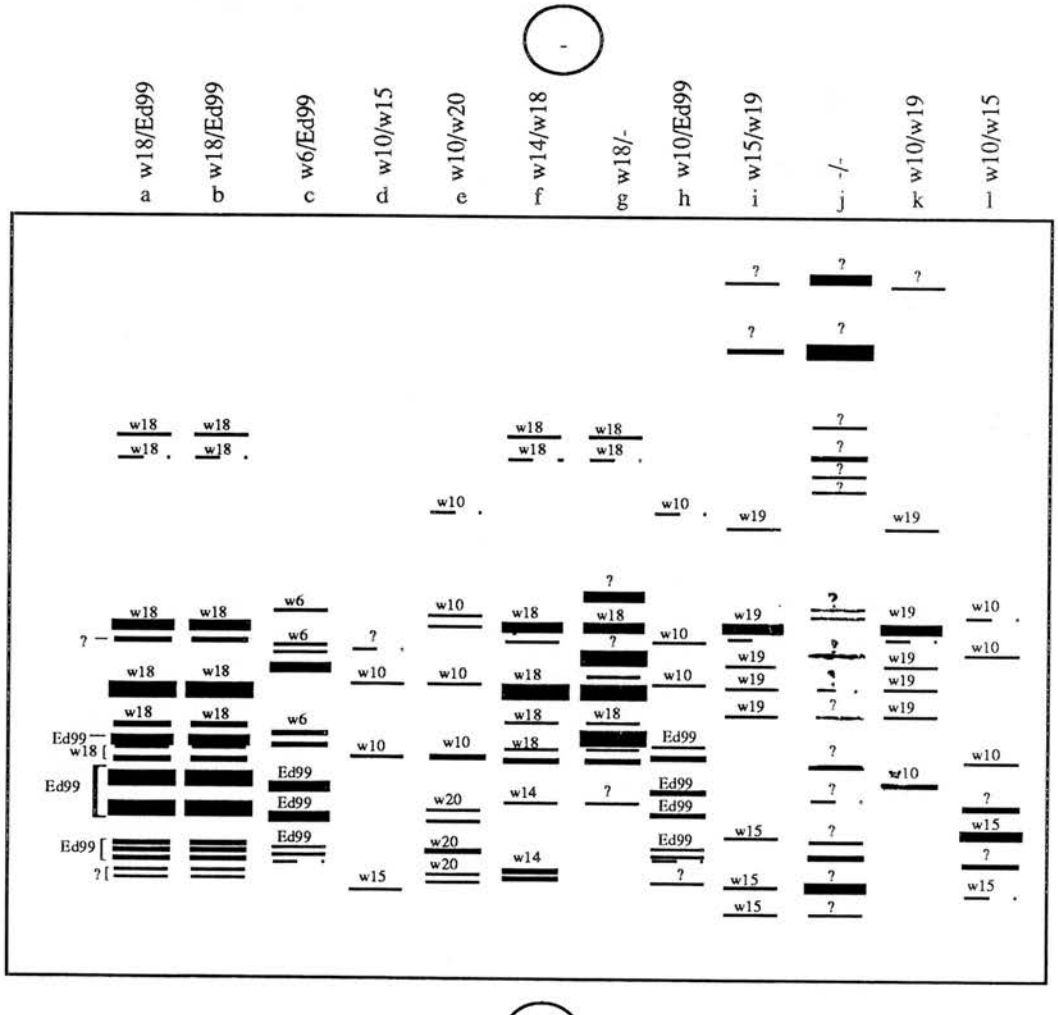

-
a w18/Ed99
b w18/Ed99
c w6/Ed99
d w10/w15
e w10/w20
f w14/w18
g w18/-
h w10/Ed99
i w15/w19
j -/-
k w10/w19
l w10/w15
+

Figure 4.17a and b, shows the 1D-IEF patterns of cells with the serological specificities w1, w2, w3, w11, w14 and two unknowns. Figure 4.17a, shows the pattern for w3 was consistent with that shown in figure 4.18 and included three bands. The w11 heavily labelled bands were also reproduced. However, only one of the two faint, more basic bands was observed. In addition to w3, lane "c" had an unknown specificity the IEF bands that might correspond to it are labelled "?". Figure 4.17b, shows the more acidic bands corresponding to the specificity w14. It also shows that the specificities w1 and w2 have seven and four IEF bands respectively, all of which had higher pIs than the w14 bands. Lane "c" in figure 4.17b also showed an unknown specificity with a single band marked "?".

Figure 4.18, shows samples that have been treated in the same way as the samples in figure 4.16. Here, the lymphocytes carried the serological specificities w3, w11, w13, w14, w20, ED99 and an unknown (-). In this figure specificities w14 and w20 were consistent with findings in figure 4.16. ED99 showed only three of the five bands allocated to it in figure 4.16, however, the labelling in that lane (d) was very poor. The specificity w11 showed a consistent pattern of two heavily labelled bands with two fainter bands, with more basic pIs only appearing on two occasions (lanes a, c and e). The serotype w13 was consistent with the presence of a single IEF band and w3 was associated with three bands. The molecules in lane "f" were precipitated from lymphocytes with serological type w14/-. The bands that correspond to this blank specificity are denoted "?".

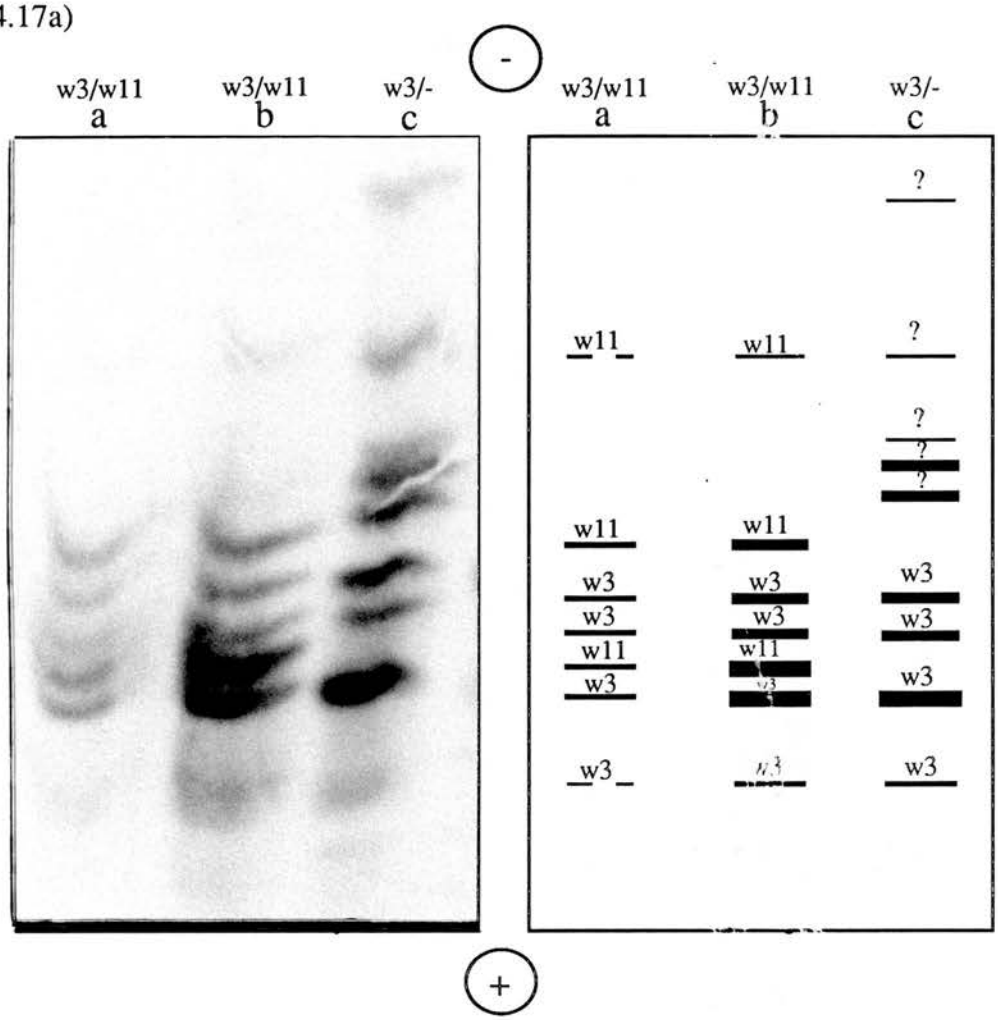
FIGURE 4.17
Facing page

COMPARISON OF IEF PATTERNS OBTAINED FROM LYMPHOCYTES WITH w2/14, w1/w14, w1/-, w3/w11, w3/w11 and w3/-.

All samples were immunoprecipitated with W6/32. Figure 4.17a the haplotypes involved were w3/w11 (lanes a and b), w3/- (lane c). The bands that could be allocated to a certain specificity are denoted with the number of that specificity. Those that could not be allocated to a certain specificity are marked "?".

In Figure 4.17b, all the samples were precipitated with MAb W6/32. The lymphocytes were from animals with serotypes, w2/w14 (lane a), w1/w14 (lane b) and w1/- (lane c).

(4.17a)


-
w3/w11
a
w3/w11
b
w3/-
c
w3/w11
a
w3/w11
b
w3/-
c
?
w11
w11
?
?
?
?
w11
w11
w3
w3
w3
w3
w11
w11
w3
w3
w3
w3
w3
w3
+

(4.17b)

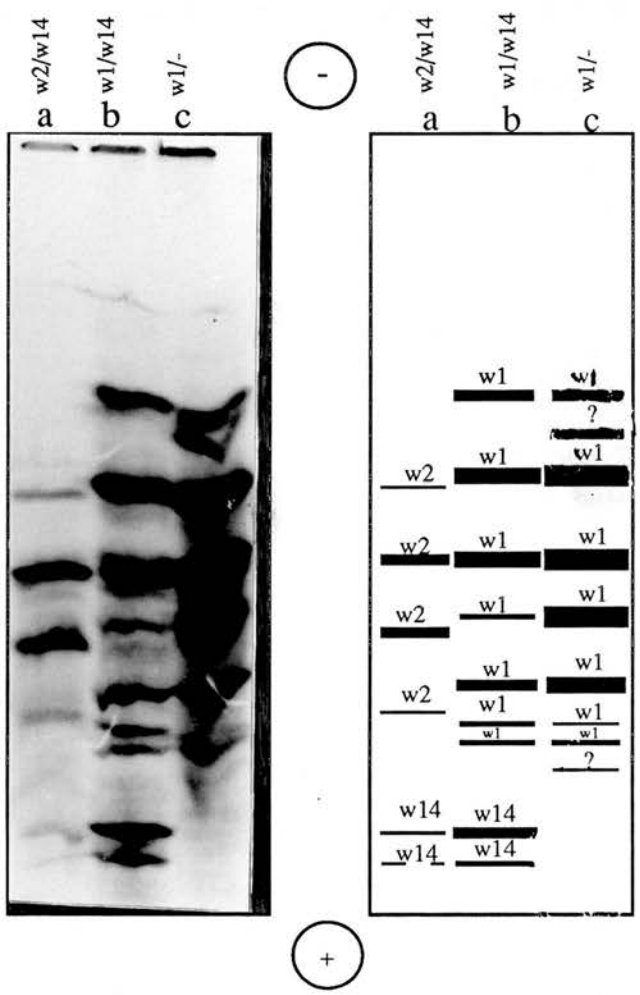
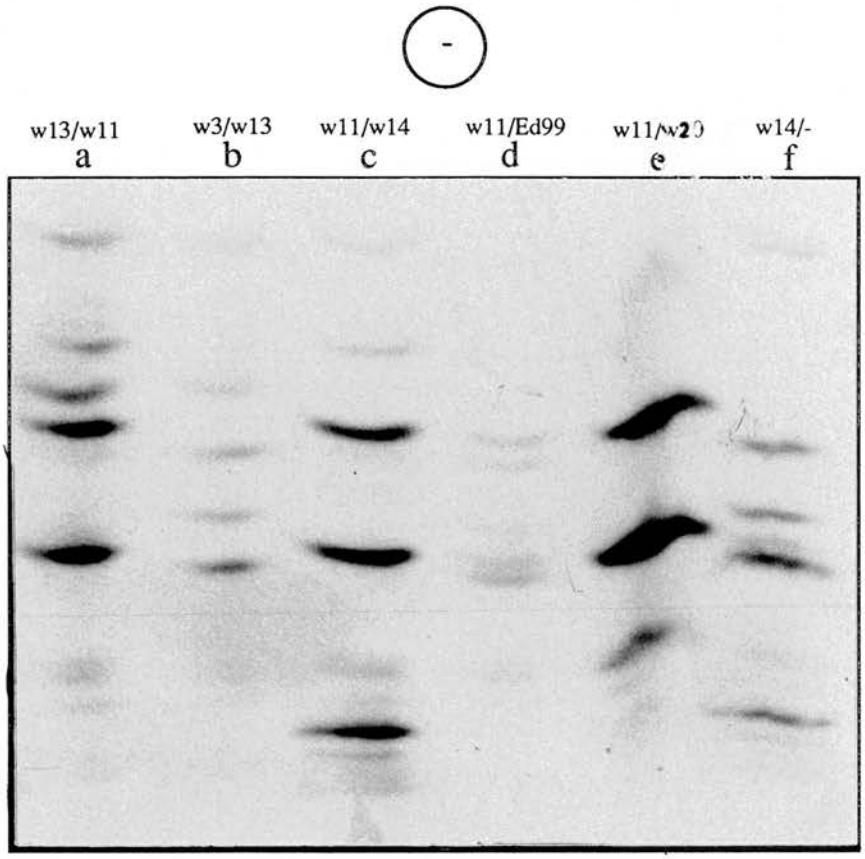

-
w2/w14
a
w1/w14
b
w1/-
c
w2/w14
a
w1/w14
b
w1/-
c
w1
w1
?
w2
w1
w1
w2
w1
w1
w2
w1
w1
w2
w1
w1
w1
w1
w1
?
w14
w14
w14
w14
+

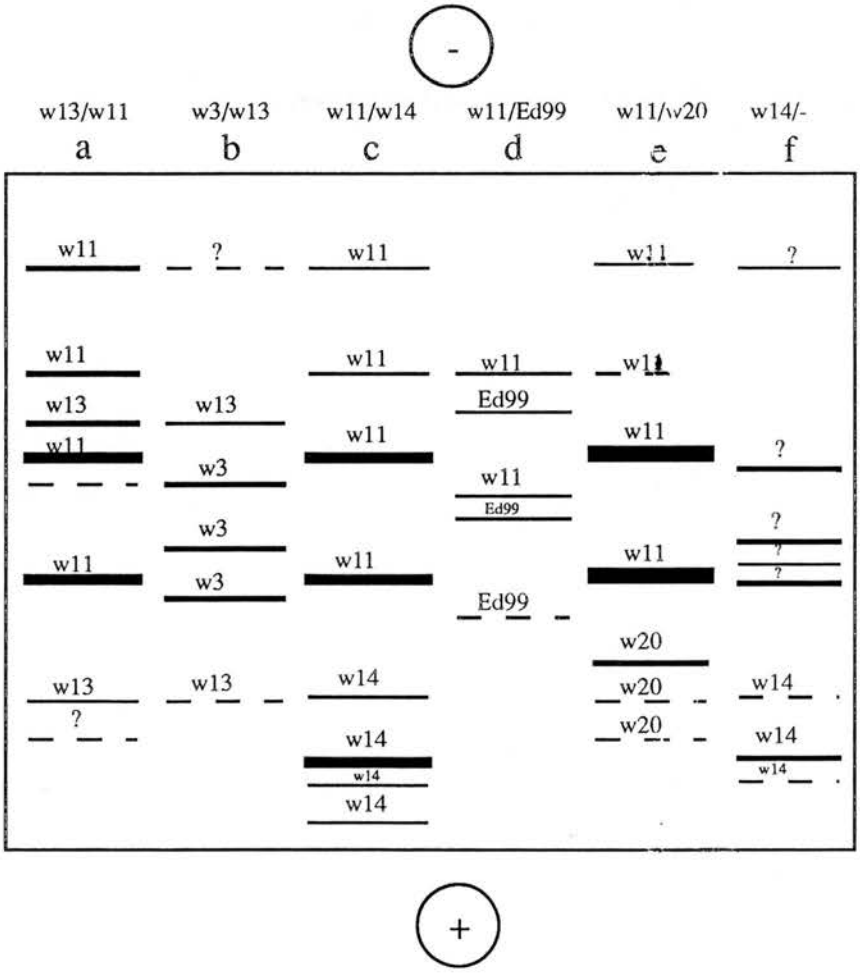
FIGURE 4.18
Facing page

COMPARISON OF IEF PATTERNS CORRESPONDING TO THE SEROLOGICAL SPECIFICITIES w3, w11, w13, w14, w20 and ED99.

All samples were precipitated with W6/32. The serological types were w11/w13 (lane a), w3/w13 (lane b), w11/w14 (lane c), w11/ED99 (lane d), w11/w20 (lane e) and w14/- (lane f).

The bands that could be allocated to a certain serological specificity are denoted with the number of that specificity. Those that could not be allocated were marked "?".


-
w13/w11
a
w3/w13
b
w11/w14
c
w11/Ed99
d
w11/w20
e
w14/-
f
+


-
w13/w11
a
w3/w13
b
w11/w14
c
w11/Ed99
d
w11/w20
e
w14/-
f
w11
?
w11
?
w11
w11
w11
Ed99
w13
w13
w11
w11
w3
w11
Ed99
w3
w11
w11
w3
w11
Ed99
w20
w13
w13
w14
w14
w20
w14
?
w20
w14
w14
w14
w14
+

These results indicated that each serological specificity is represented by a number of different charge variants that are inherited together which implies the presence of linkage disequilibrium in the BoLA system. This visual method for the allocation of IEF bands to serological specificities has good reproducibility.

A second approach to identify the different IEF bands uses the AAS in the immunoprecipitation step, Gamma-bind G agarose was used instead of Pansorbin to precipitate the AAS/class I complex, due to the formers higher ability to bind bovine Ig (McGuire, 1988). Different AAS gave banding patterns that varied in complexity. In fact some sera did not produce any bands at all. FACS analysis of the different sera suggested that the ones that were able to produce an IEF pattern had a higher proportion of IgG than IgM. Conversely the AAS which did not produce a pattern had similar proportions of IgG and IgM, and were invariably the products of a primary challenge. The results of the FACS analysis are seen in the appendix (A.2).

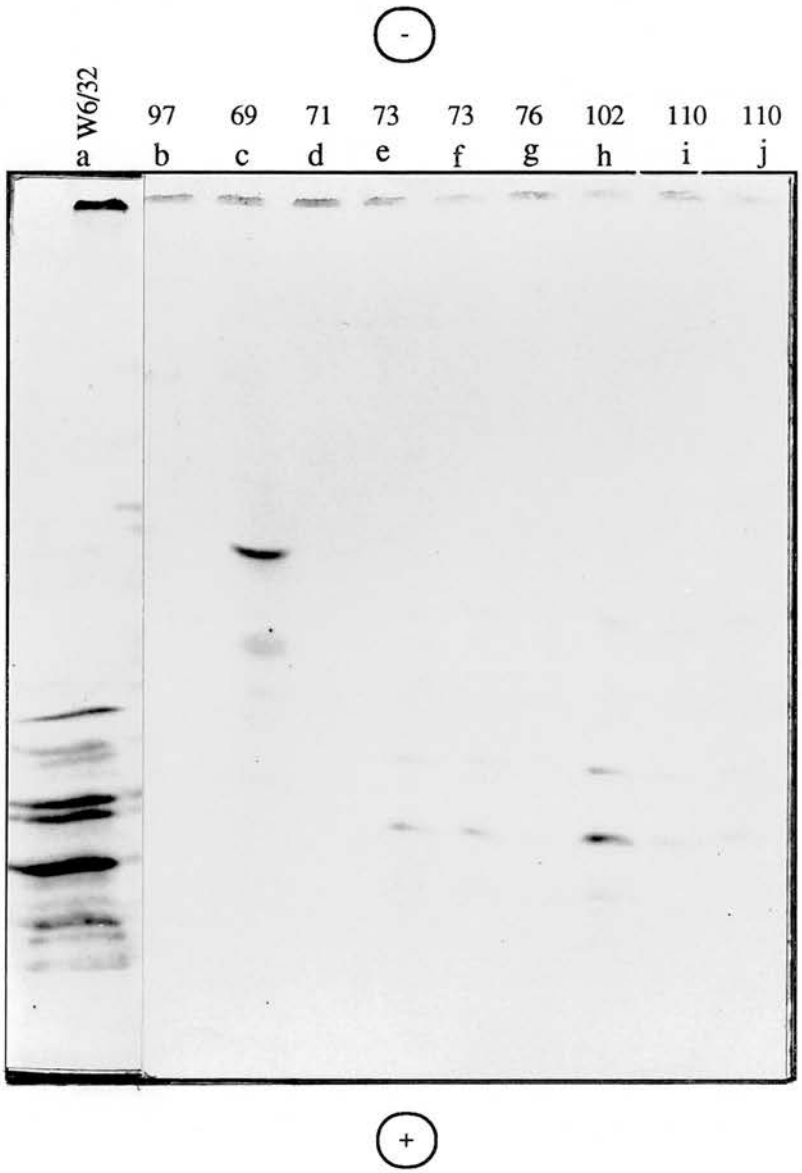
Figure 4.19 shows immunoprecipitates from an animal with the serological type w10/w11. Lane a represents the banding pattern produced using the MAb W6/32. Lane b is AAS 97 (-ve control) lanes c and d represent immunoprecipitations using the AAS 69 and 71 (w10 specific), lanes e, f, g and h represent the AAS 73, 110, 76 and 102 (w11 specific). The combination of the banding patterns of the sera of both specificities did not fully account for the IEF pattern produced with W6/32. Furthermore, the MAb recognised molecules with higher pI points (labelled "b") were not recognised by the AAS and vice versa.

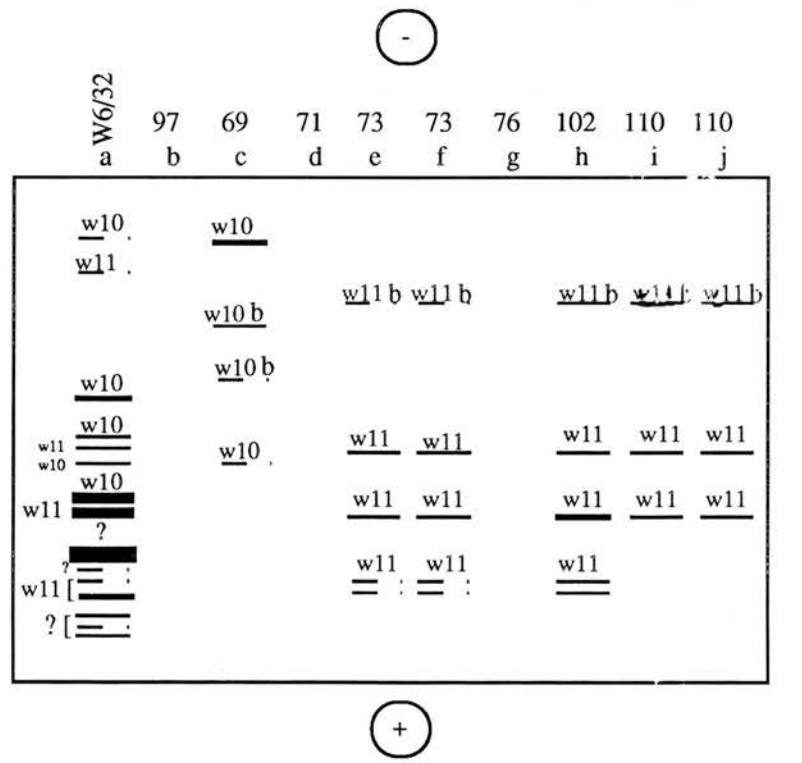
Figure 4.20a and b shows the results of immunoprecipitations from animals with the serological types w14, w15, w17, w18 and w19. The AAS corresponding to

FIGURE 4.19
Facing page

THE IEF PATTERN LYMPHOCYTES WITH THE SEROLOGICAL TYPE W10/W11 IMMUNOPRECIPITATED WITH AAS.

Lane a, is the MAb W6/32. Lane b, is the AAS 97 (w6 broad, -ve control). Lanes c and d, are the AAS 69 and 71 respectively (w10 specific). Lanes e, f, g, h, i and j are the AAS 73, 110, 76 and 102 respectively (w11 specific). The combined patterns of the two AAS did not account fully for the pattern produced with W6/32. The MAb recognised molecules not seen by any of the AAS and vice versa. The molecules recognised by the AAS but not the MAb are denoted "b".


-
W6/32
97
69
71
73
73
76
102
110
110
a
b
c
d
e
f
g
h
i
j
+


-
W6/32
97
69
71
73
73
76
102
110
110
a
b
c
d
e
f
g
h
i
j
w10
w11
w10 b
w11 b
w11
w10
?
+

the specificities w14 and w15 were not available, however, the bands corresponding to them could be assigned visually. The AAS 66 and 97 (both react to the broad specificity w6, including its subtypes w17, w18 and w19) and 100 (w19 specific) recognised a number of molecules with different pIs. These, unlike the molecules precipitated with AAS 69 and 102 (figure 4.19), were all recognised by W6/32. Furthermore, the MAb recognised molecules with more basic pIs, (denoted "b"), none of which were recognised by any of the AAS.

The results obtained above suggest that a wide range of molecules with different pI points are recognised by W6/32. The use of AAS that correspond to the serological type of any one animal did not account for the full compliment of molecules as seen with W6/32. Furthermore, in many cases there were discrepancies between the AAS and the MAb in the particular molecules recognised by each. In other words, W6/32 had the ability to recognise molecules not recognised by the AAS and vise versa. These findings suggest that class I molecules in cattle as revealed by 1D-IEF, do not behave as the products of a single highly polymorphic locus. From the above and from the fact that the majority of the animals used in these experiments were from the same breed and the same herd, the 1D-IEF patterns were identical for animals with the same serotypes. It could be suggested that there exists considerable linkage disequilibrium in the BoLA class I region. This suggestion is augmented by the fact that animals from different breeds show a distinct 1D-IEF patterns (lane j in figure 4.16). The presence of strong linkage disequilibrium would make it difficult for the AAS to distinguish among molecules expressed by the more polymorphic loci. Further, because the AAS are raised by reciprocal calf/dam immunisations the

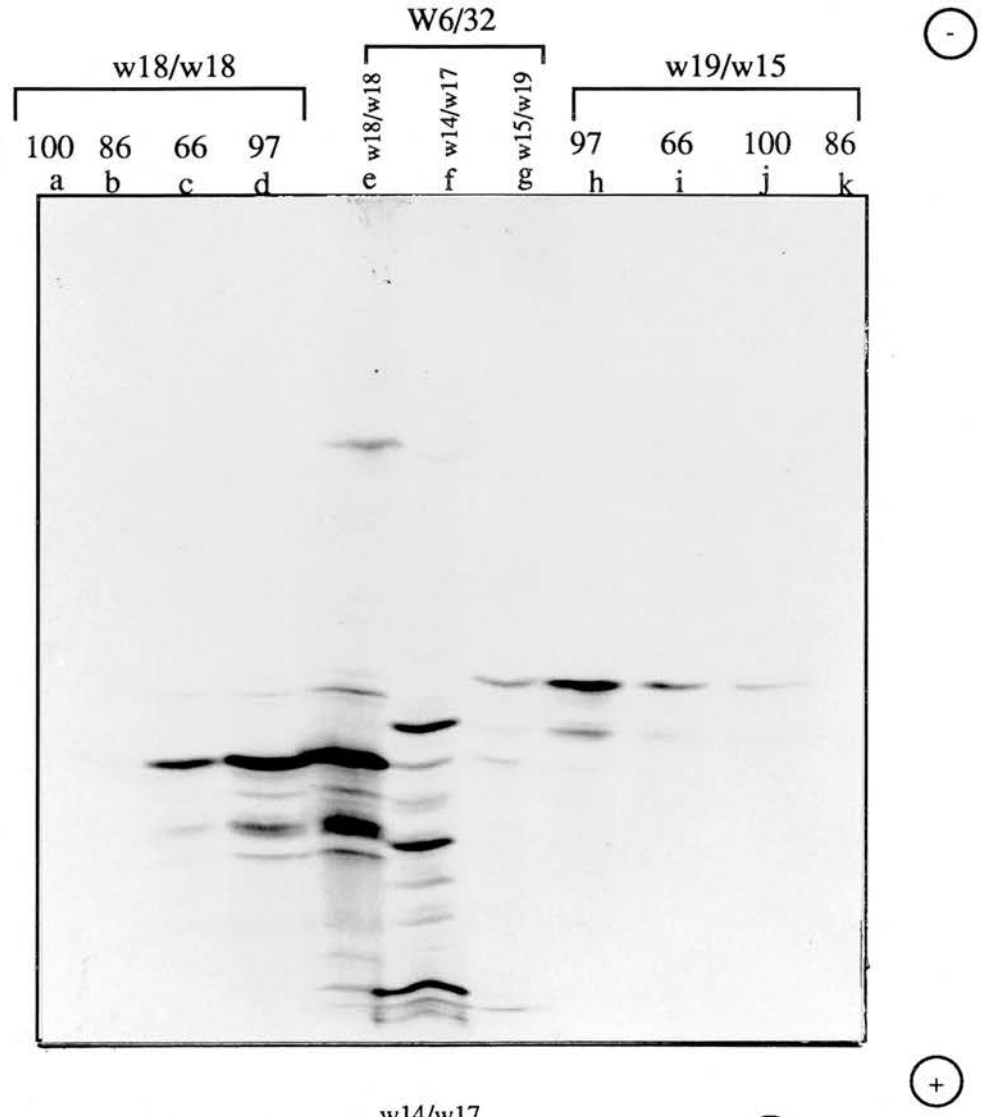
FIGURE 4.20
Next page

IMMUNOPRECIPITATIONS FROM ANIMALS WITH THE SEROLOGICAL TYPES w14, w15, w17, w18 and w19 USING AAS.

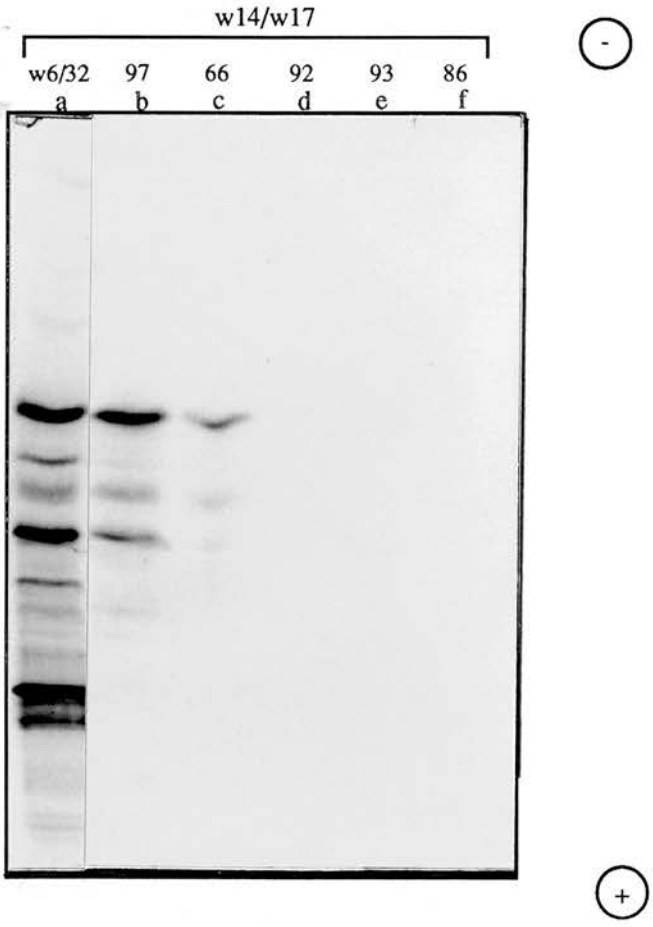
Figure 4.20a, shows a comparison of IEF patterns from animals with the serological types w18/w18 and w15/w19. Lanes a, b, and c, are lymphocytes with the serology w18/w18 precipitated with AAS a) 100 (w19 specific), as negative control, AAS 66 and 97 (broad w6) are seen in lanes b and c respectively. Lanes e, f, and g are lymphocytes with the phenotypes w18/w18, w14/w17 and w15/w19 respectively, all precipitated with W6/32. Lanes h, i, and j are w15/w19 lymphocytes precipitated with AAS 97, 66 and 100 respectively.

Figure 4.20b, shows lymphocytes with the phenotype w15/w19 precipitated with W6/32 (lane a), AAS 97 (lane b) and AAS 66 (lane c) both recognising the broad specificity w6 and its subtypes (w17, w18 and w19). Lanes d and e represent the AAS 92 and 93 (w17 specific) and lane f represents samples immunoprecipitated with AAS 86 (w18 specific) as negative control.

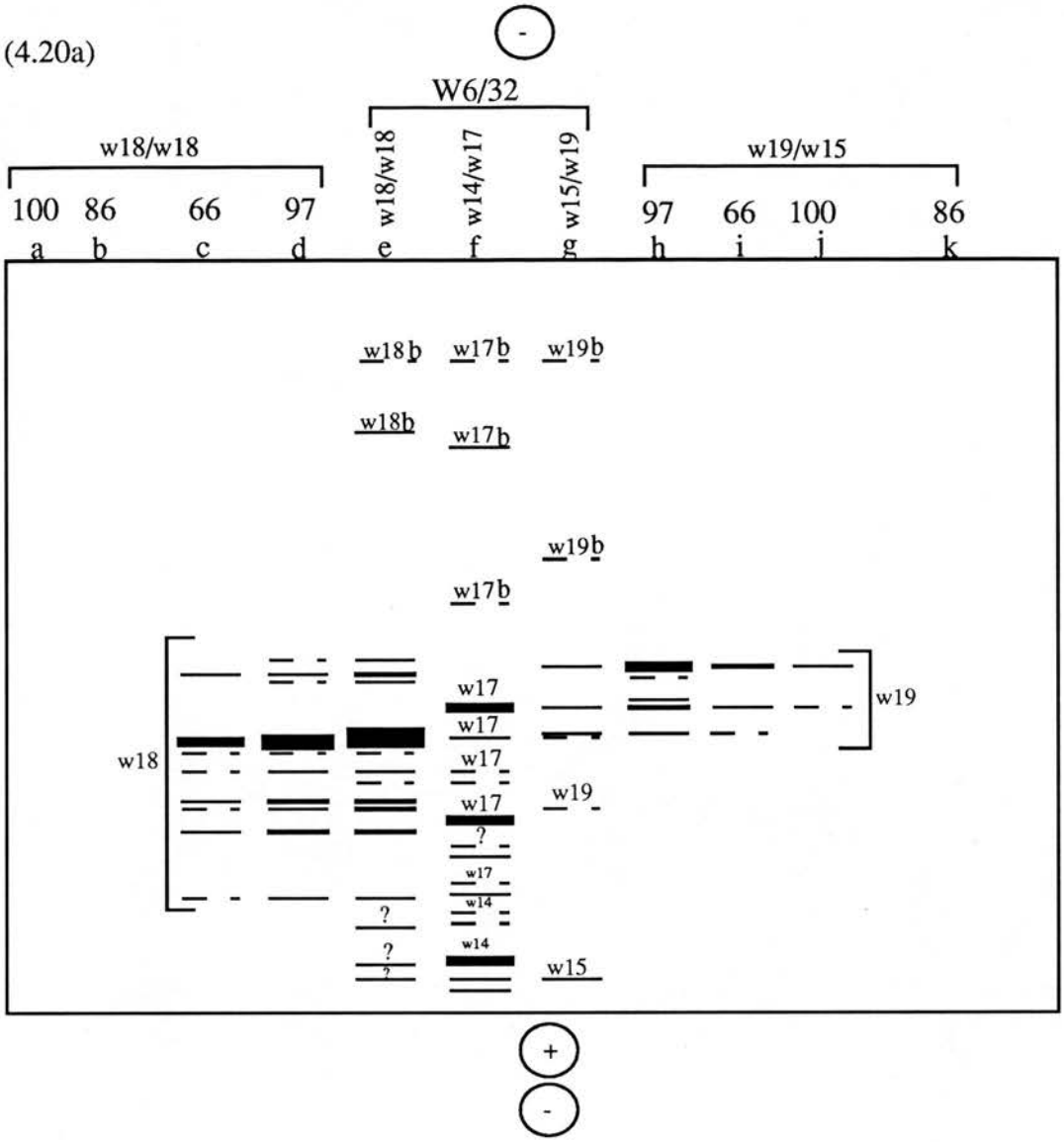
(4.20a)


W6/32
w18/w18
w19/w15
100 86 66 97
a b c d
w18/w18
w14/w17
w15/w19
e f g
97 66 100 86
h i j k
-
+

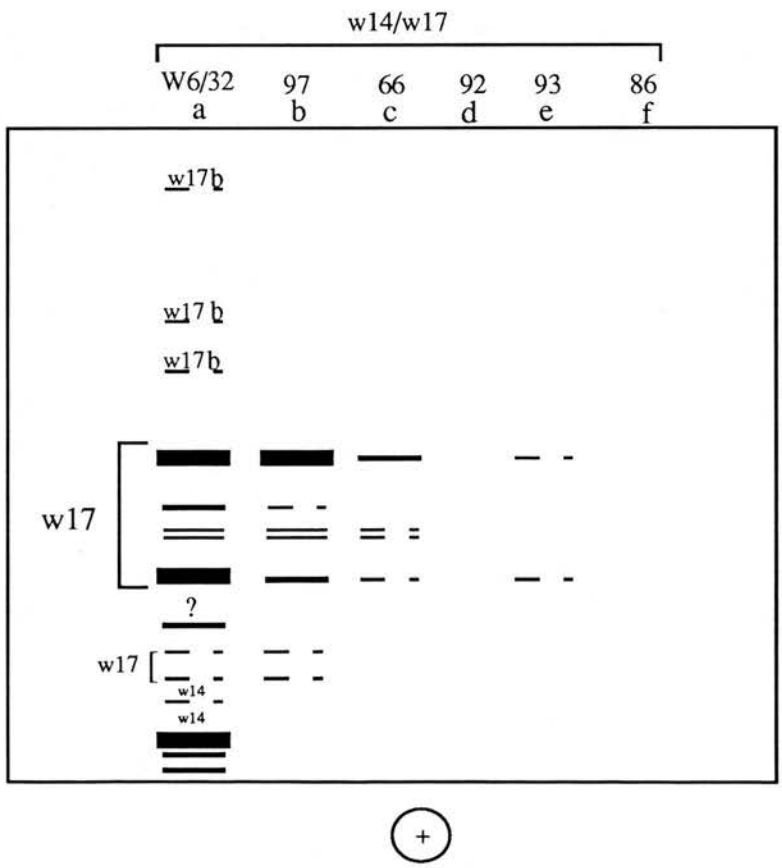
(4.20b)


w14/w17
w6/32 97 66 92 93 86
a b c d e f
-
+

(4.20a)


-
W6/32
w18/w18
w18/w18
w14/w17
w15/w19
w19/w15
100 86 66 97
a b c d
e f g
97 66 100 86
h i j k
w18b
w17b
w19b
w18b
w17b
w19b
w17b
w17
w17
w17
w17
w17
w19
w19
w18
?
?
?
w17
w14
w14
w15
+
-

(4.20b)


w14/w17
W6/32 97 66 92 93 86
a b c d e f
w17b
w17b
w17b
w17
?
w17
w14
w14
+

presence of any less polymorphic loci will be masked by this disequilibrium until animals with different genetic backgrounds are used in reciprocal immunisation procedures. Linkage disequilibrium between the class I allelic products has been reported in the human population (Bodmer and Bodmer, 1978).

4.4. STRUCTURAL COMPUTER ANALYSIS OF THE AVAILABLE BoLA CLASS I SEQUENCES.

In this section the available bovine class I sequences (Ennis et al.,1988; Brown et al.,1989 and Bensaid et al.,1991) will be subject to structural analysis using the GCG sequence analysis software. The results from this section will enable us to draw conclusions on the similarities or differences between the BoLA molecule's structure and that predicted for the HLA molecule A2.

4.4.1. Determination of the isoelectric points:

The GCG programme used in this case is called "Isoelectric". It calculates the isoelectric point of a protein from its amino acid sequence assuming no electrostatic interactions occur that perturb ionisation. "Isoelectric" makes a plot of the total positive and negative charges and the net charge of a protein as a function of pH. The isoelectric point is indicated on the plot (GCG Programme Manual).

The five sequences studied were BL3-6, BL3-7 (Ennis et al.,1988), pBoLA1 (Brown et al.,1989), KN104 and w10 (Bensaid et al.,1991). The results are shown in figures 4.21, 4.22 and 4.23. The pI points all fell in the expected range for class I

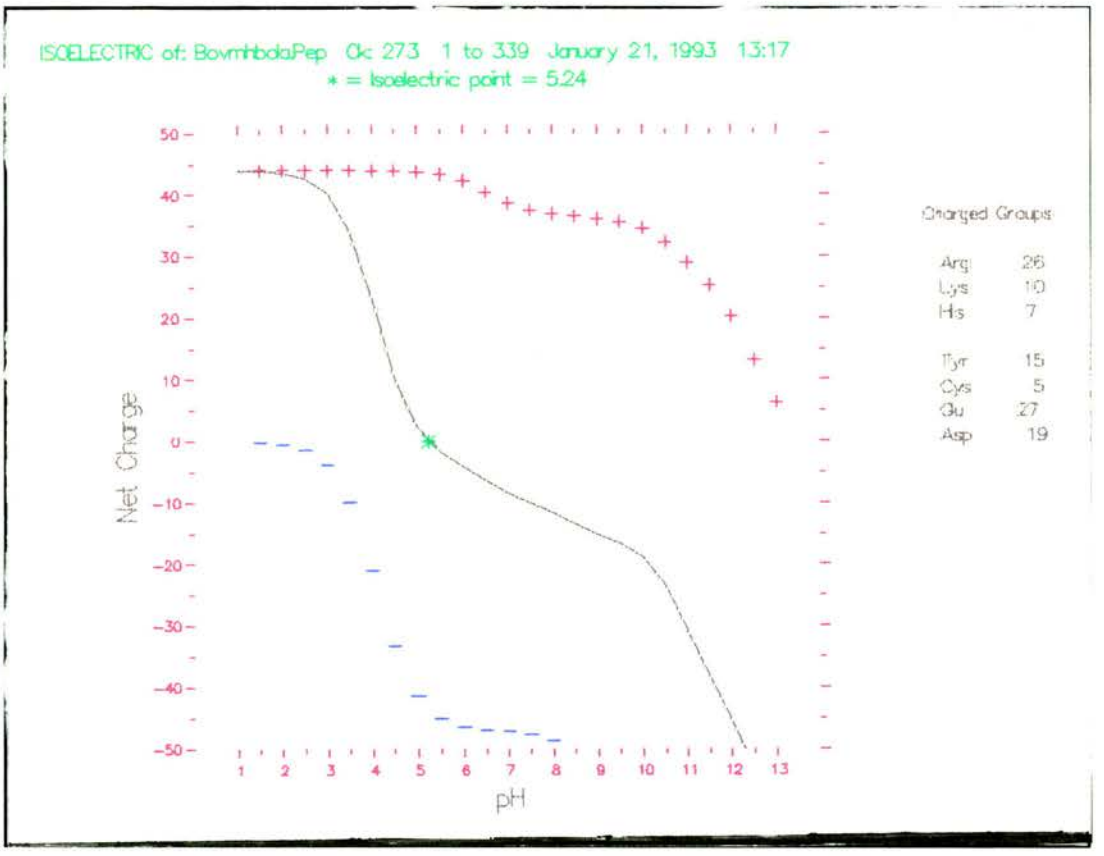
molecules at pH 5-7, with w10 having the highest pI point at 6.11 (fig. 4.22) which is consistant with the higher pIs shown by the molecules associated with w10 (section 4.3). BL3-6 had the lowest pI point at 5.24 (fig. 4.21). The IEF method used in these experiments is more than adequate in its resolution to deal with very small differences in pI points, 0.01 pH units as given by the manufacturers booklet (Pharmacia).

FIGURE 4.21
Facing page

THE PREDICTION OF THE ISOELECTRIC POINTS OF THE BoLA SEQUENCES BL-6 AND BL-7.

The isoelectric points are predicted for the sequences BL-6 (4.21a) and BL-7 (4.21b) using the GCG programme "isoelectric". The charged groups involved are given to the right of each figure. The isoelectric points were 5.24 for BL-6 and 5.27 for BL-7.

(4.21a)


ISOELECTRIC of: BovmhbolaPep Oc 273 1 to 339 January 21, 1993 13:17
* = Isoelectric point = 5.24
Net Charge
pH
Charged Groups
Arg 26
Lys 10
His 7
Tyr 15
Cys 5
Glu 27
Asp 19

(4.21b)

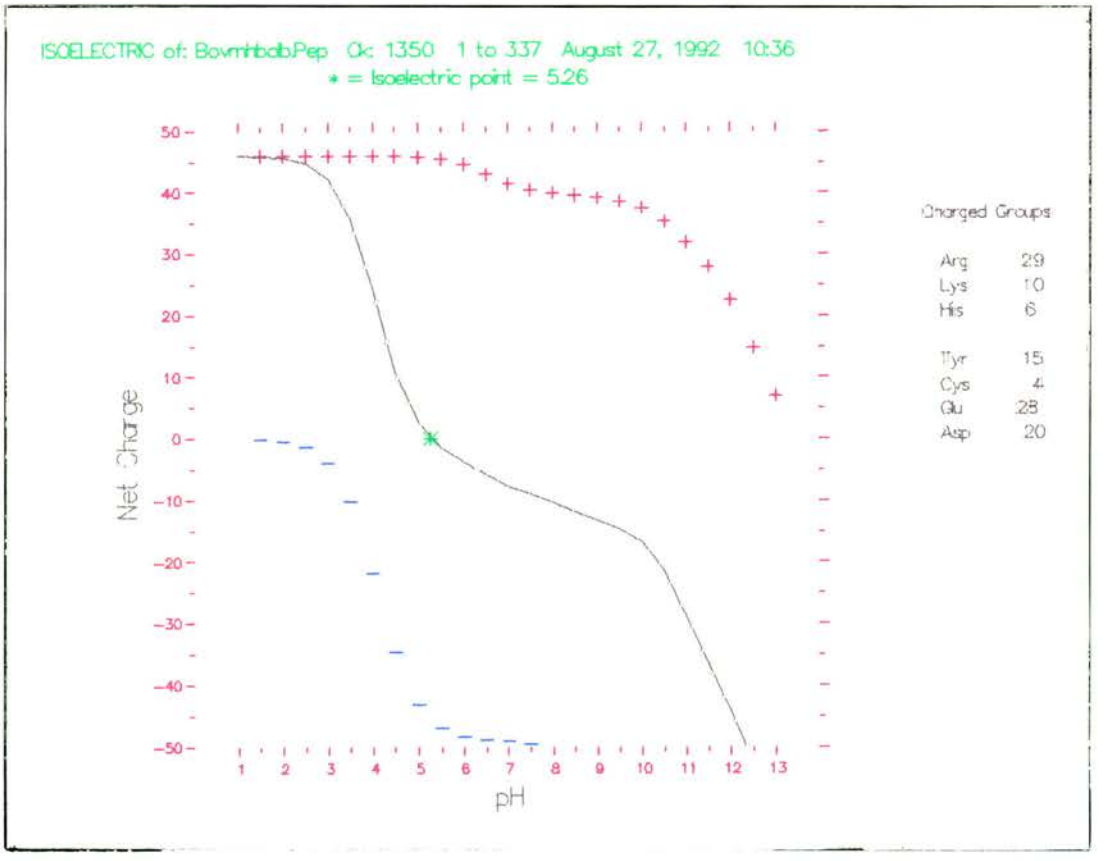
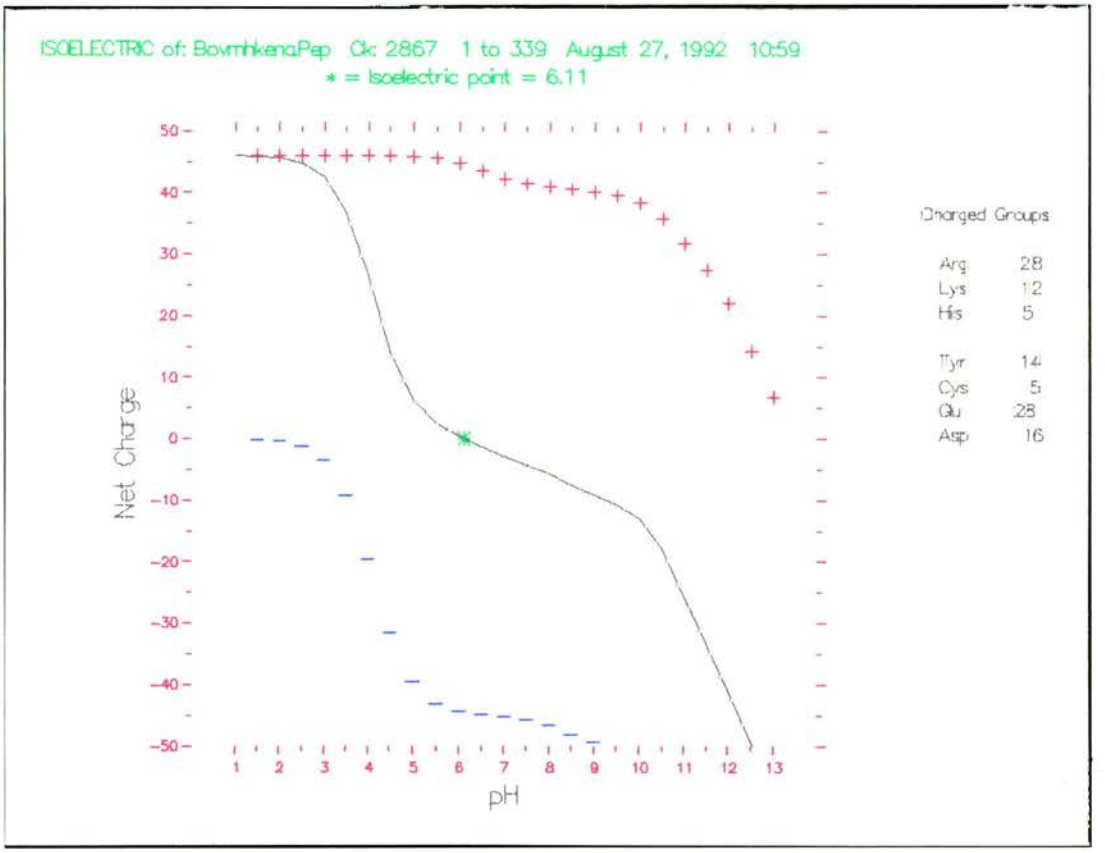

ISOELECTRIC of: BovmhbolbPep Oc 1350 1 to 337 August 27, 1992 10:36
* = Isoelectric point = 5.26
Net Charge
pH
Charged Groups
Arg 29
Lys 10
His 6
Tyr 15
Cys 4
Glu 28
Asp 20

FIGURE 4.22
Facing page

THE PREDICTED ISOELECTRIC POINT FOR THE BoLA SEQUENCES w10 and KN104.

The isoelectric points for the sequences w10 (4.22a) and KN104 (4.22b) are given using the GCG programme "isoelectric". The isoelectric points were 6.11 for w10 and 5.71 for KN104. The charged groups are given to the right of each figure.

(4.22a)


ISOELECTRIC of: BovmhkenaPep Ck: 2867 1 to 339 August 27, 1992 10:59
* = Isoelectric point = 6.11
Net Charge
pH
Charged Groups
Arg 28
Lys 12
His 5
Tyr 14
Cys 5
Glu 28
Asp 16

(4.22b)

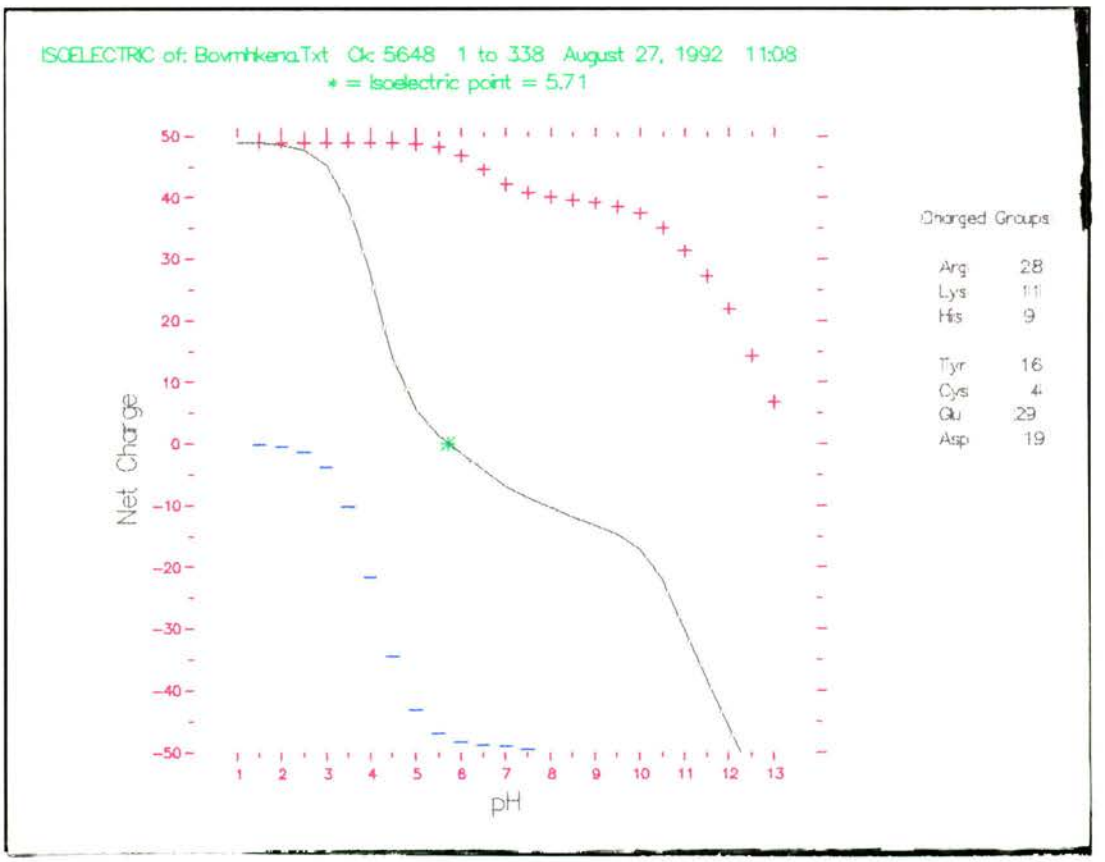
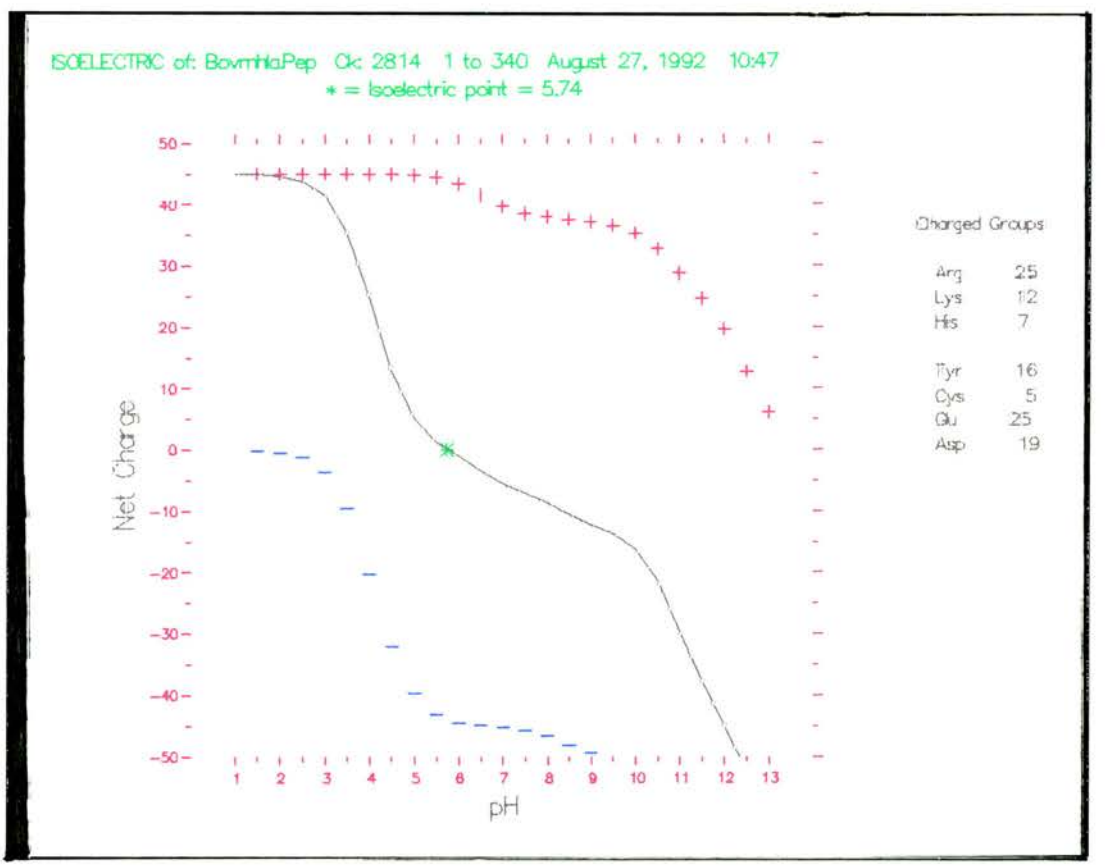

ISOELECTRIC of: BovmhkenaTxt Ck: 5648 1 to 338 August 27, 1992 11:08
* = Isoelectric point = 5.71
Net Charge
pH
Charged Groups
Arg 28
Lys 11
His 9
Tyr 16
Cys 4
Glu 29
Asp 19

FIGURE 4.23
Facing page

THE PREDICTED ISOELECTRIC POINT FOR THE SEQUENCE pBoLA1.

The predicted isoelectric point for the sequence is 5.74 as given by the GCG programme "isoelectric". The charged groups are given to the right of the figure.


ISOELECTRIC of: BovmhlaPep Cc 2814 1 to 340 August 27, 1992 10:47
* = Isoelectric point = 5.74
Net Charge
pH
Charged Groups
Arg 25
Lys 12
His 7
Tyr 16
Cys 5
Glu 25
Asp 19

4.4.2. Prediction of the secondary structure:

The secondary structure of the bovine class I sequences was predicted using the GCG programme "Peptidestructure". The secondary structure predictions made by this programme include, alpha, beta, coil, and turn, measures of antigenicity, flexibility, hydrophobicity and surface probability are also given. Details of this programme are given in the relevant section of the GCG users manual.

Peptidestructure makes predictions of the following features of an amino acid sequence:

1. Secondary structure according to the Chou-Fasman (1978) method.
2. Secondary structure according to the Garnier-Osguthorpe-Robson (1978) method.
3. Hydrophobicity according to Kyte-Doolittle (1982) method.
4. Surface probability according to the Emini (1985) method.
5. Flexibility according to the Karplus-Shulz method.
6. Glycosylation sites.
7. Antigenic index according to Jameson-Wolf (1988) method.

The basic rules for the secondary structure according to Chou-Fasman (CF) predictions are as follows: (A) For β-sheets there should be at least three β-forming residues and not more than one breaking residue within a window of five. (B) For α-helices there should be four or more α-forming residues and not more than one breaking residue within six residues. For Garnier-Osguthorpe-Robson (GOR) the predictions of the secondary structure observe the following rules: (A) The minimum length for β-sheets is four residues as opposed to five with the CF method. (B) The minimum length for an α-helix forming stretch is six residues with no restrictions.

N-linked glycosylation sites are predicted for sites where the residues have the composition NxT or NxS. When x is D, W and P, the site is taken to be a weak glycosylation site, otherwise it is a strong glycosylation site.

Caution must be taken when using these methods to interpret the structure of a given protein. As the predictions made by these programmes are only 50-60% accurate due to the fact that they rely on statisical information derived from a small number of primary sequences (Argos,1989 and Rost et al.,1993). The "peptidestructure" files are presented graphically using the GCG programme "plotstructure" and the results are given in figures 4.24-29.

The secondary structure of the HLA-A2 molecule has been reported and is used here as reference. the method of prediction used was that described by Kabsch and Sander (1983) and was found to be in agreement with the refined three-dimensional structure of the same molecule (Saper et al.,1991). The β-strands in each β sheet of each domain are referred to as Sn where n could be any number from 1-7. The α-helices are given the symbol Hn where n could be any number between 1 and 3. The HLA-A2 secondary structure could be summarized as follows (Saper et al.,1991):

The α_1 domain: NH_2-S1 (residues 3-12)-S2 (residues 21-28)-S3 (residues 31-37)-S4 (residues 46-47)-H1 (residues 50-53)-H2 (residues 57-84)-COOH.

The α_2 domain: NH_2-S1 (residues 94-103)-S2 (residues 109-118)-S3 (residues 121-126)-S4 (residues 133-135)-H1 (residues 138-150)-H2a (residues 152-161)-H2b (residues 163-174)-H3 (residues 176-179)-COOH.

The α_3 domain: NH_2-S1 (residues 186-193)-S2 (residues 198-208)-S3 (residues 214-

219)-S3s* (residues 222-224)-S4a (residues 228-230)-S4b (residues 234-235)-S5 (residues 241-250)-S6 (residues 257-262)-S7 (was not detected in the 3D structure)-COOH.

* S3s is an additional short strand running antiparallel to S3.

The predicted secondary structure for each of the five bovine sequences are given below. In each case, whenever the prediction made using this programme gives an identical or close predictions to those described above that part of the predicted structure be highlighted in bold type.

1. The predicted secondary structure of BL-6 (Ennis et al.,1988):-

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-S4 (residues 72-78)-COOH.

The α_2 domain: NH_2-S1 **(residues 108-117)**-S2 **(residues 121-128)**-H1 **(residues 135-153)**-H2 **(residues 163-173)**-S3 (residues 176-182)-COOH.

The α_3 domain: NH_2-S1 **(residues 196-203)**-H1 (residues 112-119)-S2 **(residues 243-250)**-COOH.

The transmembrane domain (TM): NH_2-S1 (residues 280-299)-H1 (residues 300-312)-COOH.

The cytoplasmic domain (C): NH_2-S1 (residues 316-323)-S2 (residues 330-339)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-15)**-S2 **(residues 20-29)**-H1 (residues 44-58)-S3 (residues 72-85)-COOH.

The α_2 domain: NH_2-S1 **(residues 91-98)**-S2 **(residues 110-119)**-H1 (residues 122-128)-S3 (residues 129-134)-H2 **(residues 135-155)**-S4 (residues 156-62)-H3 **(residues 162-171)**-H4 **(residues 175-183)**-COOH.

The α_3 domain: NH_2-H1 (residues 185-192)-S1 **(residues 195-208)**-S2 **(residues 210-219)**-H2 (residues 220-235)-S3 **(residues 237-248)**-S4 **(residues 253-262)**-H3 (residues 263-268)-S5 (residues 269-274)-COOH.

The TM domain: NH_2-S1 (residues 280-308)-COOH.

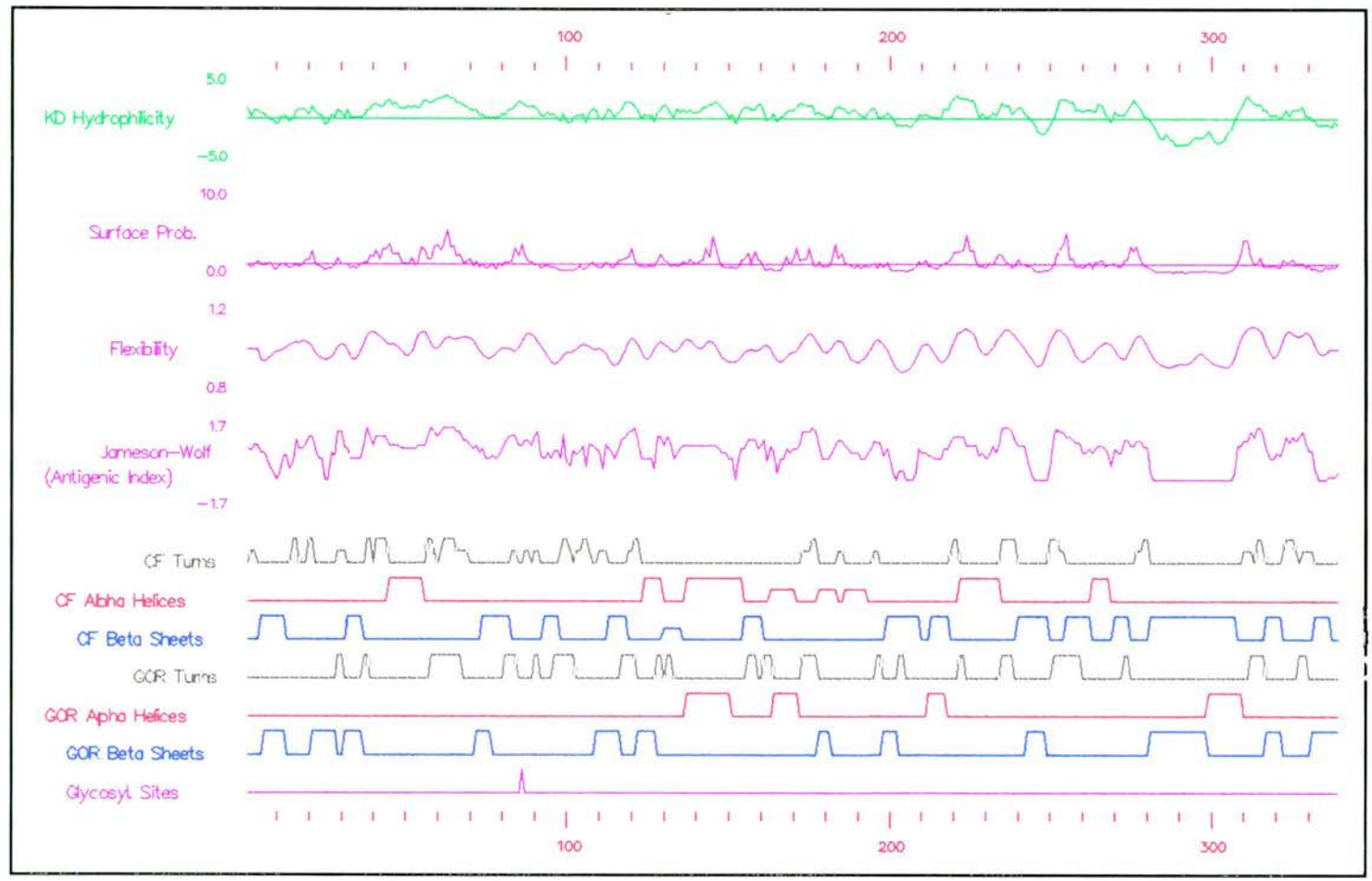
The C domain: NH_2-S1 (residues 316-323)-S2 (residues 330-336)-COOH.

The diagram representing the above is given in figure 4.24. The figure also shows the existence of a single N-linked glycosylation site at position 86. Additionally, the sequence of residues 281-307 was shown to be hydrophobic and represents the position of the transmembrane domain.

FIGURE 4.24
Facing page

THE PREDICTED SECONDARY STRUCTURE OF THE BoLA SEQUENCE BL-6.

The general characteristics of the secondary structure of BL-6 as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Apha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

2. The predicted secondary structure of BL-7 (Ennis et al.,1988):

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-S4 (residues 72-78)-COOH.

The α_2 domain: NH_2-S1 **(residues 94-102)**-S2 **(residues 108-113)**-H1 (residues 125-145)-H2 **(residues 145-158)**-H3 **(residues 165-175)**-S3 (residues 177-182)-COOH.

The α_3 domain: NH_2-COOH.

The TM domain: NH_2-S1 (residues 281-195)-H1 (residues 296-307)-COOH.

The C domain: NH_2-S1 (residues 314-320)-S2 (residues 328-337)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 47-57)**-S4 (residues 65-76)-S5 (residues 76-83)-COOH.

The α_2 domain: NH_2-S1 **(residues 94-102)**-S2 **(residues 108-118)**-H1 (residues 125-160)-H2 **(residues 165-171)**-H3 **(residues 179-184)**-COOH.

The α_3 domain: NH_2-H1 (residues 185-196)-S1 **(residues 200-210)**-H2 (residues 211-239)-S2 **(residues 240-250)**-S3 **(residues 254-264)**-H3 (residues 264-270)-S4 (residues 271-276)-COOH.

The TM domain: NH_2-S1 (residues 281-307)-COOH.

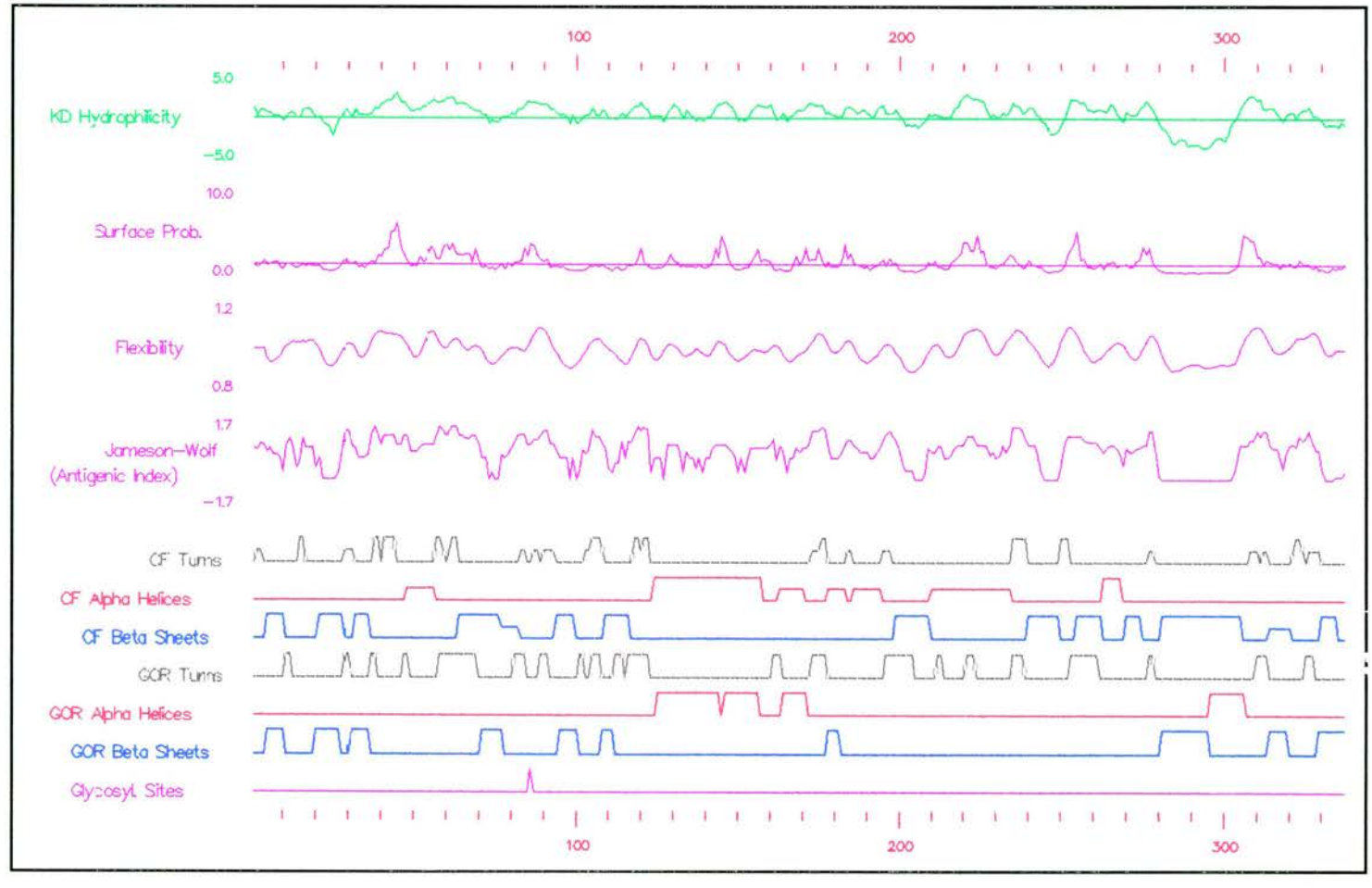
The C domain: NH_2-S1 (residues 314-320)-S2 (residues 328-335)-COOH.

The transmembrane domain extends from residues 280-303 which are shown to be hydrophobic. Furthermore, position 86 is the only glycosylation site observed as seen in figure 4.25.

FIGURE 4.25
Facing page

THE PREDICTED SECONDARY STRUCTURE OF THE BoLA SEQUENCE BL-7.

The general characteristics of the secondary structure of BL-7 as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

3. The predicted secondary structure of pBoLA1 (Brown et al.,1989):

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 57-66)**-S4 (residues 70-76)-S5 (residues 80-85)-COOH.

The α_2 domain: NH_2-S1 **(residues 111-117)**-S2 **(residues 121-129)**-H1 **(residues 130-147)**-H2 (residues 148-157)-H3 **(residues 165-173)**-S3 **(residues 175-182)**-COOH.

The α_3 domain: NH_2-S1 **(residues 198-203)**-S2 **(residues 225-235)**-COOH.

The TM domain: NH_2-S1 (residues 281-297)-H1 (residues 298-312)-COOH.

The C domain: NH_2-S1 (residues 317-323)-S2 (residues 331-339)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-15)**-S2 **(residues 31-37)**-H1 **(residues 50-58)**-H2 **(residues 64-71)**-S3 (residues 72-87)-COOH.

The α_2 domain: NH_2-S1 **(residues 92-98)**-S2 **(residues 111-119)**-H1 **(residues 123-155)**-S3 (residues 162-172)-H2 **(residues 176-182)**-COOH.

The α_3 domain: NH_2-H1 (residues 186-192)-S1 **(residues 196-209)**-S2 **(residues 212-218)**-H2 (residues 221-232)-H3 (residues 240-250)-S3 **(residues 254-263)**-H4 (residues 264-269)-H5 (residues 270-276)-COOH.

The TM domain: NH_2-S1 (residues 281-309)-COOH.

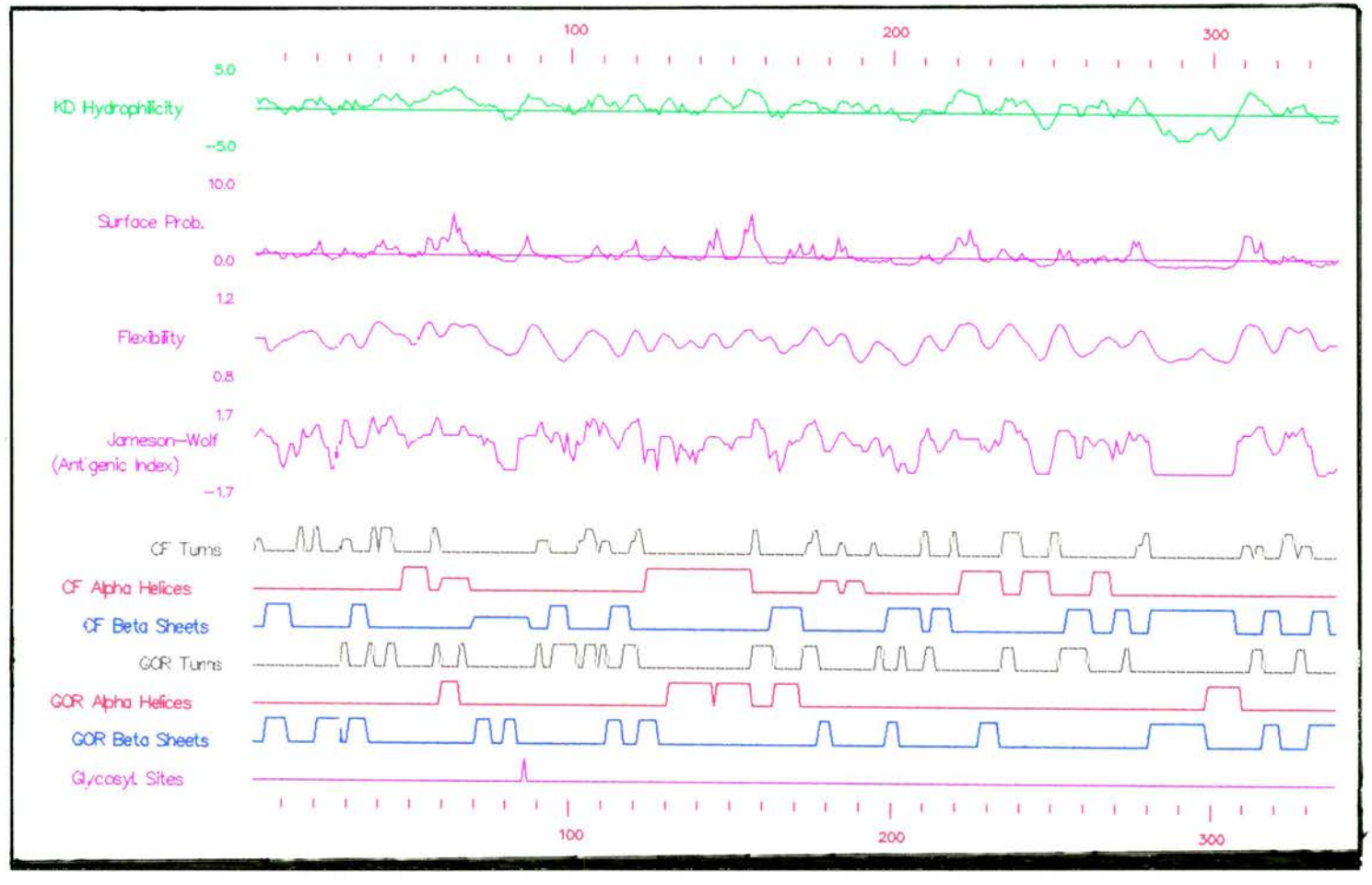
The C domain: NH_2-S1 (residues 315-323)-S2 (residues 330-339)-COOH.

The hydrophobic region extended from residues 281-307 and there was a single glycosylation at position 86. These results are given in figure 4.26.

FIGURE 4.26
Facing page

THE PREDICTED SECONDARY STRUCTURE OF THE BoLA SEQUENCE pBoLA1.

The secondary structure of the sequence pBoLA1 as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

4. The predicted secondary structure for a w10 molecule (Bensaid et al.,1991):

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 60-67)**-S4 (residues 70-78)-COOH.

The α_2 domain: NH_2-S1 **(residues 111-117)**-H1 **(residues 124-158)**-H2 **(residues 165-173)**-S2 **(residues 177-183)**-COOH.

The α_3 domain: NH_2-S1 **(residues 186-193)**-S2 **(residues 197-204)**-S3 **(residues 226-234)**-S4 **(residues 259-264)**-COOH.

The TM domain: NH_2-S1 (resides 280-298)-H1 (residues 299-309)-COOH.

The C domain: NH_2-S1 (residues 316-223)-S2 (residues 330-339)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-15)**-S2 **(residues 31-37)**-H1 **(residues 44-58)**-H2 **(residues 60-72)**-S3 (residues 70-78)-COOH.

The α_2 domain: NH_2-S1 **(residues 93-103)**-S2 **(residues 111-117)**-H1 (residues 121-158)-S3 (residues 159-165)-H2 **(residues 166-174)**-H3 **(residues 177-183)**-COOH.

The α_3 domain: NH_2-H1 (residues 185-191)-S1 **(residues 197-209)**-S2 **(residues 212-218)**-H2 (residues 220-234)-H3 (residues 239-249)-H4 (residues 253-268)-S3 (residues 269-274)-COOH.

The TM domain: NH_2-S1 (residues 280-309)-COOH.

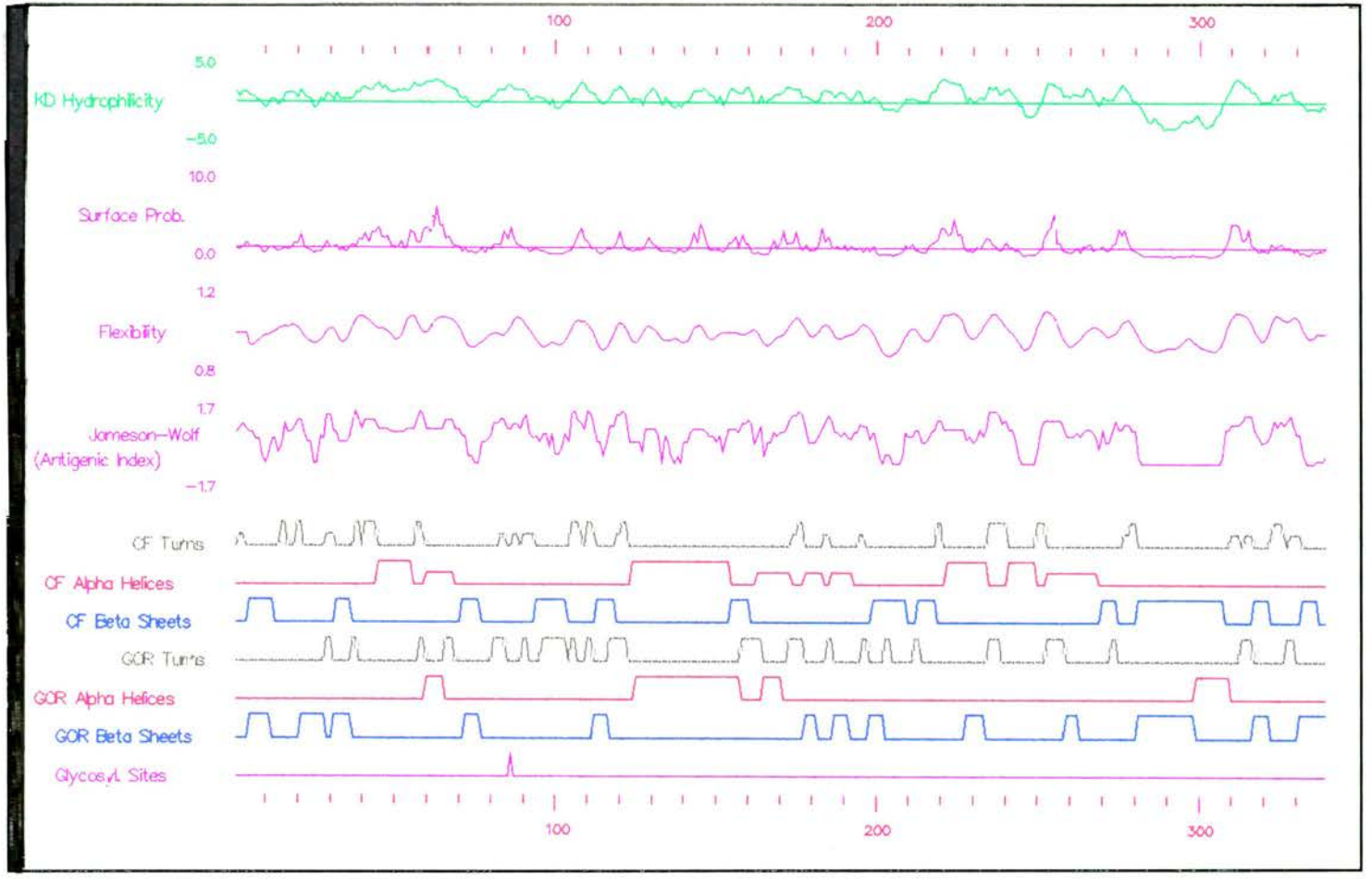
The C domain: NH_2-S1 (residues 316-223)-S2 (residues 330-337)-COOH.

The hydrophobic residues extend over position 281-307 and there is a single glycosylation site at position 86 as seen in figure 4.27.

FIGURE 4.27
Facing page

THE PREDICTED STRUCTURE OF THE BoLA SEQUENCE w10.

The secondary structure of the a sequence which is part of the w10 haplotype as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

5. The predicted secondary structure of KN104 (Bensaid et al.,1991):

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 47-54)**-H2 **(resides 60-76)**-COOH.

The α_2 domain: NH_2-S1 **(residues 106-112)**-S2 **(residues 122-128)**-H1 (residues 134-144)-H2 **(residues 164-173)**-COOH.

The α_3 domain: NH_2-S1 (residues 177-183)-H1 (residues 194-203)-H2 (resides 210-219)-S2 **(residues 240-250)**-COOH.

The TM domain: NH_2-S1 (residues 280-299)-H1 (residues 300-309)-COOH.

The C domain: NH_2-S1 (residues 315-323)-S2 (residues 330-338)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-13)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 43-55)**-H2 **(residues 59-70)**-S4 (residues 71-76)-S5 (residues 78-85)-COOH.

The α_2 domain: NH_2-H1 **(residues 90-97)**-S1 **(residues 121-129)**-H2 **(residues 133-150)**-S2 (residues 155-161)-H3 **(residues 163-169)**-COOH.

The α_3 domain: NH_2-H1 (residues 176-184)-H2 (residues 185-196)-S1 **(residues 197-208)**-S2 **(residues 211-219)**-H3 (residues 221-234)-S3 **(residues 240-259)**-S4 **(residues 254-264)**-H4 (residues 265-268)-S5 (residues 269-274)-COOH.

The TM domain: NH_2-S1 (residues 280-308)-COOH.

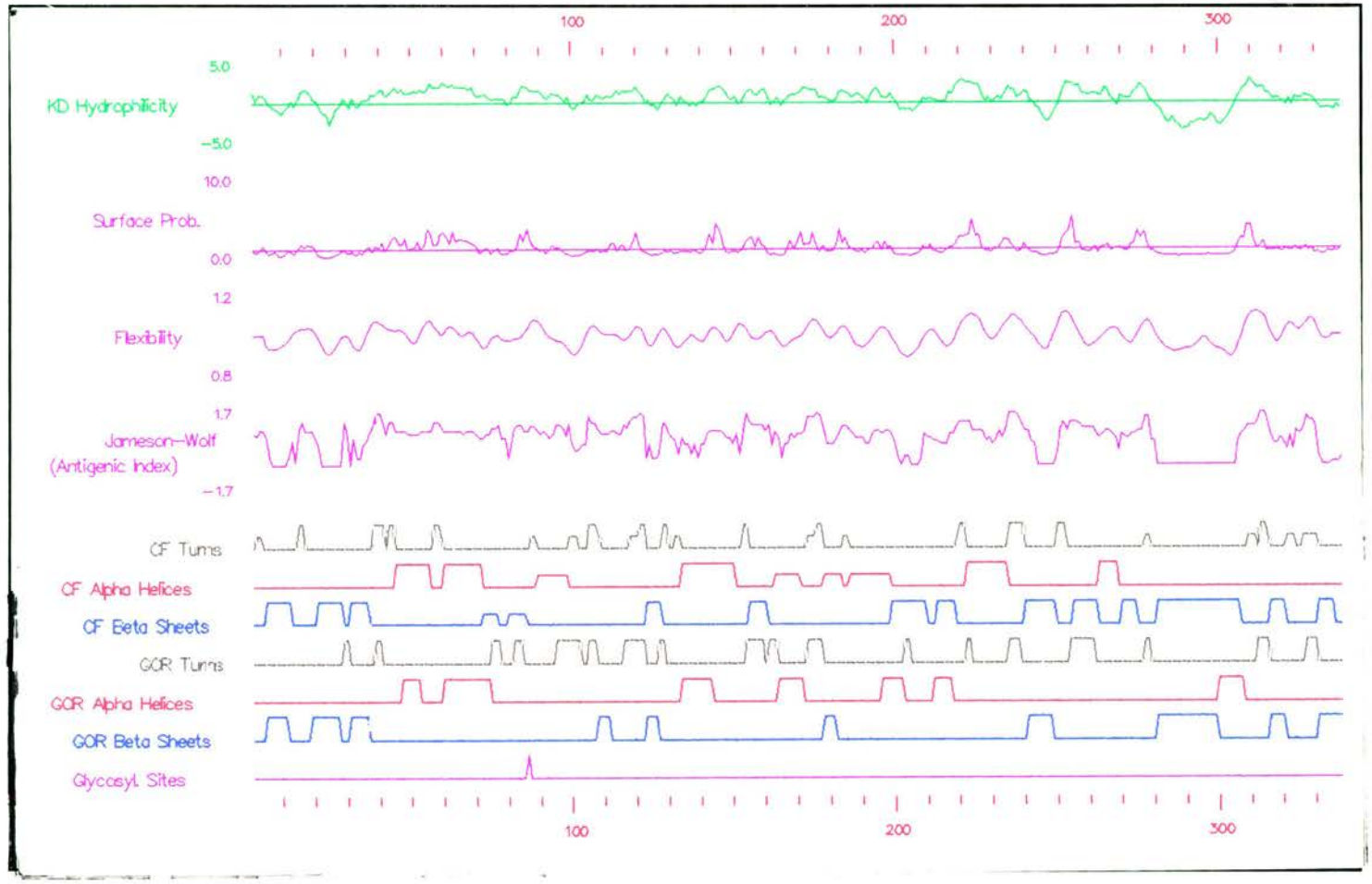
The C domain: NH_2-S1 (residues 314-320)-S2 (residues 330-338)-COOH.

The hydrophobic region extended between residues 281-305 and there was a single N-linked glycosylation site at position 86 as shown in figure 4.28.

FIGURE 4.28
Facing page

THE PREDICTED SECONDARY STRUCTURE OF THE BoLA SEQUENCE KN104.

The secondary structure of the sequence KN104 as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

6. The predicted secondary structure of HLA-A2 (Koller and Orr,1985):

(a) The secondary structure using the GOR method:

The α_1 domain: NH_2-S1 **(residues 5-12)**-S2 **(residues 20-29)**-S3 **(residues 31-37)**-H1 **(residues 60-71)**-S4 (residues 72-82)-COOH.

The α_2 domain: NH_2-S1 **(residues 95-100)**-H1 **(residues 122-160)**-H2 **(residues 163-173)**-COOH.

The α_3 domain: NH_2-H1 (residues 185-205)-S1 **(residues 228-234)**-S2 **(residues 243-250)**-COOH.

The TM domain: NH_2-S1 (residues 280-299)-H1 (residues 300-309)-COOH.

The C domain: NH_2-S1 (residues 318-324)-S2 (residues 336-341)-COOH.

(b) The secondary structure using the CF method:

The α_1 domain: NH_2-S1 **(residues 5-15)**-S2 **(residues 20-30)**-S3 **(residues 31-37)**-H1 (residues 40-49)-H2 **(residues 50-58)**-H3 **(residues 65-79)**-S4 (residues 80-89)-COOH.

The α_2 domain: NH_2-S1 **(residues 94-99)**-S2 **(residues 106-119)**-H1 **(residues 122-160)**-S3 (residues 161-175)-COOH.

The α_3 domain: NH_2-H1 (residues 178-185)-H2 (residues 186-196)-S1 **(residues 197-210)**-S2 **(residues 211-218)**-H3 (residues 226-235)-S3 **(residues 240-250)**-S4 **(residues 254-264)**-S5 (residues 266-273)-COOH.

The TM domain: NH_2-S1 (residues 280-310)-COOH.

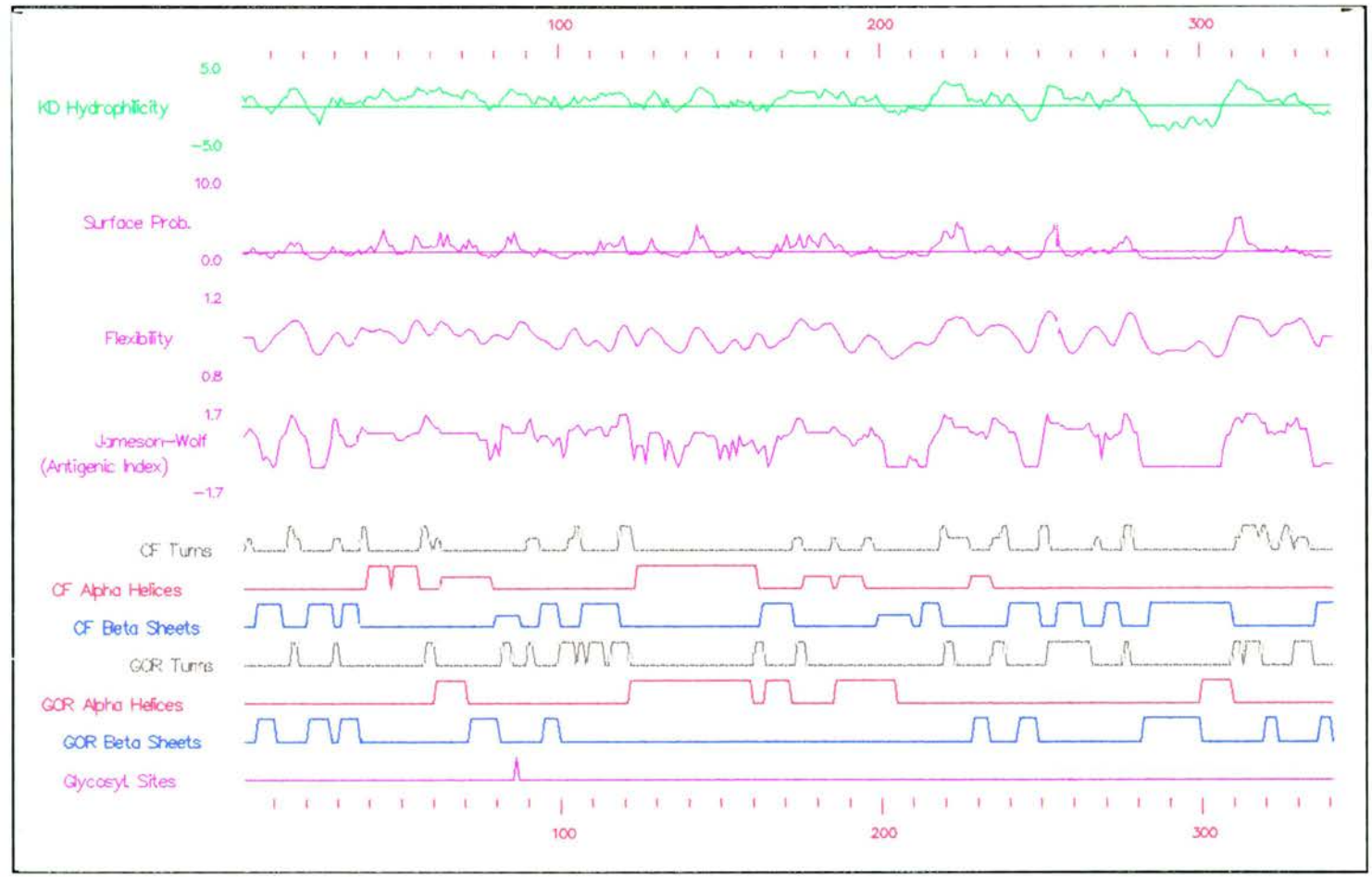
The C domain: NH_2-S1 (residues 333-341)-COOH.

The hydrophobic region extended between residues 281-307 and there was a single N-linked glycosylation site a position 86 as shown in figure 4.29.

FIGURE 4.29
Facing page

THE PREDICTED SECONDARY STRUCTURE FOR THE HLA-A2 MOLECULE.

The secondary structure of the human MHC sequence A2 as predicted using the GCG programme "peptidestructure".


100
200
300
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300

The above predictions, although relying on "peptidestructure" which is an error prone programme, suggest that the large majority of the bovine sequences in the α_1, α_2 and α_3 domains form α-helices or β-strands at positions similar (bold type) to those predicted for the HLA-A2 molecule using a more accurate programme (Saper et al.,1991). In fact, when the HLA-A2 secondary structure was predicted using "peptidestructure", it was found to be similar to those of the BoLA sequences, and showed similar deviations from the more accurate secondary structure reported by Saper and co-workers (1991). It could be argued that the secondary structure of the BoLA class I molecules is similar to that of HLA molecules and it is therefore likely that the same applies for their three-dimensional structures. The glycosylation site at position Asn 86, for both BoLA and HLA molecules was predicted accurately using the programme "peptidestructure". The transmembrane region was also accurately predicted. The use of this programme, although useful in determining the structural similarities between the BoLA and HLA molecules was not sensitive enough with the present information to determine whether the various BoLA sequences reflected locus specific structural characteristics.

4.4.3. Comparison of the published BoLA class I sequences in the TM domain:

In the MHCs of humans and mice all the alleles encoded by the same locus have the same number of amino acid residues in their transmembrane domais (TM). The only exception to this rule are H-2K molecules, the TM regions of which contain 39 or 40 residues (Ennis et al.,1988).

The reported BoLA class I sequences show three different groups of TM domains with 37, 36 and 35 residues, respectively as is shown below:

```
BL-6:     EPPQPSFLTMGIIVGLVLLVVTGAVVAGVVICMKKRS
BoL1:     EPPQPSFLTMGIIVGLVLLVVTGAVVAGVVICMLLRS
w10 :     EPPQPSFLTMGIIVGLVLLVVTGAVVAGVVICMKKRS
19.2:     EPPQPSFLTMGIIVGLVLLVVTGAVVAGVVICMKKRS
19.1:     EPPQTSFLTMGIIVGLVLLVVTGVVVAGAVIWMKRRS
K104:     EPPQTSFLTMGIIVGLVLL-VTGAVVAGFVIWMKKRS
BL-7:     EPPQTSFLIMGIIVGLVLLVV--ALVAGAVIWRKKRS
19.3:     EPPQTSFLTMGIIVGLVLLVV--AVVAGAVIWRKKRS
19.4:     EPPQTSFLIMGIIVGLVLLVV--AVVAGAVIWRKKRS
```

The polymorphic positions in each sequence are highlighted. It is clear that the sequences containing 35 residues (BL-7, 19.3 and 19.4) all lack two amino acids (T and G) from the same positions, which are conserved in the other six sequences, indicating that they are likely to be encoded by the same stretch of DNA. The sequence KN104, on the other hand, lacks a single amino acid from a different position. From the above sequences, it is clear, that if the number of amino acid residues in the TM domain is a specificity of the encoding locus, then the BoLA class I molecules are encoded by at least three distinct class I loci. These findings are in agreement with the rest of the results presented in this chapter.

CHAPTER FIVE

Discussion and Conclusions

5. DISCUSSION AND CONCLUSIONS:

Until very recently the bovine MHC was studied solely by serology. AAS reacting to polymorphic epitopes, raised by calf/dam reciprocal immunisations are used to determine the different specificities expressed in a particular animal. Using this technique it has been generally accepted that the bovine MHC encodes a single highly polymorphic class I locus (Anon,1982; Bull et al.,1989; Bernoco et al.,1992). This came from the observation that in most cases each animal could only be identified by a maximum of two AAS with no evidence of recombination (Bull et al.,1989; Bernoco et al.,1992).

The use of sensitive techniques such as 1D-IEF, that goes beyond the resolution of serology, produce complex pictures of the bovine MHC class I encoding region (Joosten et al.,1988; Oliver et al.,1989). In each case, a multiple band pattern was produced from individual animals by immunoprecipitating the class I molecules with MAbs that recognise monomorphic epitopes on the class I molecules.

The aim of this study was to determine biochemically the origins of the expressed charge heterogeneity observed with 1D-IEF of the class I molecules obtained from non-stimulated bovine peripheral blood lymphocytes. The study set out to establish the extent to which different post-translational modifications affect the observed heterogeneity, and to estimate the number of expressed class I loci in normal, non-stimulated bovine PBL.

Recent biochemical and molecular biological studies involving the BoLA system have suggested the presence of two expressed class I loci. Ennis and co-

workers (1988) reported the primary sequences of two bovine cDNA clones and from the deduced nucleotide sequences it was suggested that these clones (BL3-6 and BL3-7) could not be allelic products of the same locus. This finding was based on the observation that the 3' untranslated regions in humans and mice of alleles from the same locus show a mean homology of 96+/-2.3%, whereas homology between alleles of different loci is in the range 74-95%. The 3' untranslated regions of BL3-6 and BL3-7 have a homology of 86.2% which is in the range of alleles of different loci (Ennis et al.,1988). In another report DNA from an animal expressing the BoLA types w10 and KN104 was digested with rare cutter enzymes (Sfi I, Mlu I, Not I and Cla I) and fragments were size separated using field inversion gel electrophoresis (FIGE). Two cDNA clones (2.1 and 5.1) were isolated from a cDNA library prepared from the same animal, these two clones showed divergence at the both termini. Hybridisation with an entire class I cDNA probe revealed multiple fragments generated by each of the enzymes used. When the 3' untranslated regions of 2.1 and 5.1 were used as probes, only a single fragment was revealed in each digested sample, showing the locus specificity of these probes. DNA from transfected mouse L cells, expressing either KN104 or w10 (Toye et al.,1990), hybridised to the 3' untranslated regions of clones 2.1 and 5.1, respectively. Northern blot analysis of the mRNA of the two transfected cell lines provided further evidence that the cDNA clones 2.1 and 5.1 code for molecules of the BoLA-KN104 and BoLA-w10 serotypes, and they are representative of two different loci. The long range physical mapping of these genes using FIGE analysis of DNA from homozygous as well as heterozygous animals revealed that they are separated by not more than 210kb (Bensaid et al.,1991a).

Bensaid and co-workers (1991b) also reported that the rare-cutter enzyme Sfi I cleaves the DNA from an animal which is serologically typed as w10/KN104 into six fragments. As it was assumed that each BoLA class I gene measures 5kb, and that the probability of there being an Sfi I site in any given 5kb piece of DNA is low, it is probable that every Sfi I fragment contains at least one class I gene, this is exemplified by the finding that two distinct, 3' untranslated regions are transcribed by the same Sfi I fragment of 250kb (Bensaid et al.,1991a). Thus there is the possibility of the presence of at least seven BoLA class I loci (Bensaid et al.,1991b). Further evidence for the presence of at least two class I BoLA loci came from the use of the polymerase chain reaction to amplify cDNA from expressed bovine MHC class I genes. Sequences obtained from transmembrane and cytoplasmic domains were used to identify the number of expressed alleles. The results suggested the presence of four alleles representing the products of two (or more) class I loci. The data obtained also suggested the presence of alternatively spliced mRNA which results in the removal of exon 7, predicting a truncated molecule with the cytoplasmic portion 16 amino acids shorter than usual (Ellis et al.,1993). It must be emphasised that the experiments described by Ellis and co-workers involved the use of Theileria annulata transformed peripheral blood mononuclear cells. Such activated cells have been shown to express activation-associated class I molecules which are not present on normal cells. The amino acid backbones of such molecules were shown to exhibit a wide range of molecular masses (M_r) including M_r of approximately 37,000, which is 2,000-3,000 less than the estimated M_r of the amino acid backbones of normal class I molecules (Bensaid et al.,1989). This size heterogeneity observed by Bensaid and coworkers

(1989) could reflect the expression of alternatively spliced class I molecules as described by Ellis and co-workers (1993). Ulker and co-workers (1990) reported that concanavalin A activated T cells express almost exclusively alternatively spliced forms of the class Ib molecule Qa-2.

Biochemical evidence for the expression of two expressed BoLA class I loci came from studies carried out by Joosten and co-workers (1992). In these experiments a polymorphic monoclonal antibody (IL-A31), raised against a molecule of an African w10 specificity, recognised a product that was distinct from other w10 associated molecules precipitated with W6/32 as was revealed by peptide mapping using the endoprotease V8. Therefore it was concluded that the serologically defined w10 serotype encodes at least two independent class I locus products expressed on normal bovine PBM (Joosten et al.,1992).

5.1. THE EXPRESSED BOVINE CLASS I MOLECULES:

SDS-PAGE analysis of the immunoprecipitates revealed that all MAbs recognised a doublet with heavy and light variants at 43,000-44,000 and 39,000-40,000 respectively (Fig. 4.1), Both variants were sensitive to neuraminidase treatment indicating that they have transversed the trans-Golgi network. Their presence in the detergent phase indicated that they still retain their hydrophobic regions suggesting that they are membrane bound. The molecules exhibiting the molecular mass between 43,000-44,000 are most likely to represent mature class I molecules. The lighter molecules are artifacts of the immunoprecipitation procedure. This was confirmed by

the use of protease inhibitors in the lysis buffer which lead to a dramatic reduction in the presence of such molecules (Fig. 4.8a). The complexity of the 1D-IEF patterns obtained from samples treated with protease inhibitors using MAb W6/32 was unchanged (Fig. 4.8b). This indicated that the presence of the light molecular mass variants in samples not treated with protease inhibitors did not contribute significantly to the observed charge heterogeneity and that other factors were exerting their influence. The results from the 2D SDS-PAGE analysis give support to this suggestion by revealing that most, although not all, the charge variants observed on the first dimension subsequently separate into heavy and light molecular mass variants on the second dimension. The reason for the light molecular mass variants retaining the same charge as their parent molecules could be explained by inspecting the five published class I sequences (Ennis et al.,1988; Brown et al.,1989 and Bensaid et al.,1991). In all these class I sequences, the cytoplasmic tail, which is the most likely part of the molecule to be digested, harbours equal numbers of positively and negatively charged residues. Therefore, it could be envisaged that the removal of this part of the molecule would not interfere with the overall charge of the remaining sequence. Both parent and truncated molecules, therefore, could be expected to have the same charge on 1D-IEF. On the other hand, the finding that both heavy and light variants retain the same pI point could reflect the possibility that the cytoplasmic regions of the class I molecules in our samples do not have any charged residues. Therefore, the proteolytic removal of the corresponding sequence would not have any effect on the electrophoretic migration of the rest of the molecule.

The 1D-IEF patterns produced by IL-A88 was different from that produced by W6/32 and B1.1G6 in that it revealed the presence of more basic molecules (IL-A88$^+$ in fig. 4.3). Sequential immunoprecipitation experiments revealed that the size of the IL-A88$^+$ molecules is approximately 39,000 (Fig. 4.4a). The inability of MAbs W6/32 and B1.1G6 to precipitate similar molecules could be given a number of explanations. The IL-A88$^+$ molecules might not be associated with β_2m which is required for recognition by W6/32 and B1.1G6 or, on the other hand, they could simply lack the appropriate epitope whether on the heavy (W6/32) or light chain (B1.1G6). Heavy chains that lack the association with β_2m are more prone to proteolytic digestion (Ploegh et al.,1979; Kissonerghis et al.,1980) which would explain the smaller sizes of the IL-A88$^+$ molecules. Such molecules also show transient expression at the cell surface (Ortiz-Navarrete and Hammerling,1991; Rock et al.,1991) which would explain the poor reproducibility of the IL-A88$^+$ pattern. The combination of observations of the lower molecular mass, the inability to be recognised by MAbs W6/32 and B1.1G6 and the poor reproducibility of the more basic pattern with IL-A88 itself, could be a reflection of the fact that these molecules are class I heavy chains that escaped association with β_2m during biosynthesis, or have dissociated from it at the cell surface. The existence of such products has been reported in other species (Rock et al.,1991; Vitiello et al.,1990; Hanson et al.,1988). Until recently, such surface expressed heavy chains were thought to be functionless. However, Glas and co-workers (1992) suggested that allospecific and restricted CD8$^+$ T cells capable of killing β_2m-deficient cells could be generated, indicating that surface expressed naked heavy chains can act as antigen or antigen presenting molecules.

It must be emphsised at this point that the IL-A88$^+$ molecules could also be non-MHC class I molecules that co-precipitate when the MAb IL-A88 is used.

5.2. EFFECTS OF POST-TRANSLATIONAL MODIFICATIONS ON BoLA HETEROGENEITY:

The charge heterogeneity of BoLA class I molecules observed by 1D-IEF could have resulted from the differential modification of a small number of polypeptides. Two post-translational modifications (glycosylation and phosphorylation) and of one post-transcriptional modification (alternative splicing of pre-mRNA) that might lead to the same gene product having different charges have been reported to occur in class I molecules. The effects of these modifications were studied by the use of enzymatic digestion, differential radioactive labelling with $^{32}P_i$ and ^{35}S methionine, and analysis by SDS-PAGE, 1D-IEF and 2D electrophoresis.

The sialic acids are a diverse family of 9-carbon carboxylated sugars usually found as terminal monosaccharides of animal oligosaccharides. The most common sialic acid is N-acetyl-neuraminic acid which is thought to be the precursor of all modified forms of the sugar many of which exist in ungulates (Varki,1992). The nature of the sialic acid attached to bovine MHC molecules is unknown. However, the use of neuraminidase from Vibrio cholerae reduces the heterogeneity of the 1D-IEF patterns (Fig. 4.4b). The treatment also has an effect on the migration of these molecules in SDS-PAGE. This type of neuraminidase hydrolyses O-ketosidic α2-3, α2-4, α2-6 and α2-8 bonds of terminal N-acetylneuraminic acids. Methods using this enzyme were developed in this laboratory in conjunction with Joosten and co-workers

and has been shown to completely desialate bovine MHC class I molecules (Joosten et al.,1988). Some types of sialic acids (4-O-acetylated sialic acids) are resistant to all known sialidases (Schauer,1987), however, there is no published evidence to date that suggests the presence of these sugars on MHC molecules.

The differential sialation of a single class I polypeptide could result in it having different mobilities on 1D-IEF, thus contributing to the observed heterogeneity. SDS-PAGE analysis of the neuraminidase treated samples revealed a shift in mobility that corresponds to a 1,000-1,500 change in molecular mass (Fig. 4.5). The size of the shift indicates that the BoLA class I molecules carry up to 3 sialic acid residues. The 1D-IEF analysis of these samples revealed that the treatment with neuraminidase reduces the observed charge heterogeneity and causes a shift to more basic pI points indicating the loss of a negative charge which we attribute to the loss of sialic acid residues (Fig. 4.4b). When the samples were treated with EndoF the shift was larger and reflected a decrease in molecular mass of about 4,000 (Fig. 4.5). These results indicate that the class I molecules have a single N-linked glycosylation site that is occupied by a complex sugar molecule. It was also observed that the heavy and light molecular mass variants were still present after digestion with both enzymes, suggesting that the lighter molecules in the pattern are actually fully glycosylated and that their lower molecular masses are not a reflection of incomplete glycosylation. This gives further support to the idea of them being heavy chains that have undergone proteolytic digestion at or near the cell surface. The 1D-IEF patterns of EndoF treated samples were similar to those obtained after neuraminidase treatment alone (Fig. 4.6) giving further support to the notion that the presence of sialic acids is the major

contributor to charge heterogeneity and that the digestion with neuraminidase from V. cholerae removes all heterogeneity arising from sialic acid. The findings also indicate that the BoLA class I molecules are unlikely to carry any O-linked glycosylation sites due to the fact that the molecular mass calculated for the naked amino acid backbones of the published class I sequences were approximately 40,000 (Ennis et al.,1988; Brown et al.,1989 and Bensaid et al.,1991). This, when taken together with the molecular mass contributed by the N-linked sugar molecule (4,000), would give an approximate molecular mass of 44,000 which is that of a mature class I molecule.

It must be noted at this point that the shifts observed with SDS-PAGE and 1D-IEF of samples treated with neuraminidase and EndoF are unlikely to be the products of contaminating protease activity. This stems from the fact that there was no evidence of non-specific digestion which is expected if there was active protease digestion. Furthermor, the enzymes used were of high purity and that any protease activity that could have been present was below the limits of detection as is specified by the manufacturers. However, the buffers used did not include any protease inhibitors and although proteolytic digestion is highly unlikely as was seen by 2D analysis and it could not be dismissed completely.

The class I molecules also carry phosphorylation sites at serine residues in the cytoplasmic tail of the molecule (Ellis et al.,1993). Phosphorylated class I molecules may exhibit a more acidic pI than non-phosphorylated molecules, therefore, the differential phosphorylation of a small number of amino acid backbones could be responsible for the observed 1D-IEF heterogeneity (Loube et al.,1983).

In order to investigate these options two approaches were used. In the first, non-stimulated PBM were labelled with either ^{35}S methionine or $^{32}P_i$. The cells were then lysed in a buffer containing both phosphatase and protease inhibitors and the class I molecules were immunoprecipitated in the usual manner. The SDS-PAGE and 1D-IEF analysis of the samples revealed that it was only the molecules labelled with ^{35}S methionine that were observed with no trace of any phosphorylated molecules (Figs. 4.8a,b). To determine whether the cells had actually taken up $^{32}P_i$, total lysate of phosphorylated cells was analysed using SDS-PAGE. It was clear that a number of proteins in the molecular mass range of 20,000-80,000 were phosphorylated (Fig. 4.7).

The second approach was an indirect one, in which, ^{35}S methionine labelled, neuraminidase treated class I molecules were further treated with the enzyme potato acid phosphatase (PAP). The rationale was that treatment with PAP should remove any phosphate residues from the amino acid backbone and that this will result in shifts in the electrophoretic mobility and subsequent reduction in the complexity of the patterns observed. Results from both SDS-PAGE and 1D-IEF (Figs. 4.9 and 4.10 respectively) revealed that none of the class I molecules in the pattern were affected by this treatment, and that the complexity of the 1D-IEF pattern remained identical to that obtained after neuraminidase treatment alone. These observations when taken in combination strongly suggest that the BoLA class I molecules immunoprecipitated from the membranes of resting cells are not phosphorylated. Similar finding have been reported for HLA class I molecules (Loube et al.,1983; Eichholtz et al.,1992). Therefore, the charge heterogeneity observed is not explained by differential phosphorylation events.

Another mechanism that could result in different isoforms of a single molecule is the alternative splicing of pre-mRNA. Alternative splicing is a post-transcriptional modification that could occur in almost every exon. However, pre-mRNA splices have been rarely shown to occur at the 5' end of the encoding RNA and even when such splice products have been found no corresponding protein was present (Lalanne et al.,1985 and Trancy et al.,1984). This form of alternative splicing could add to the diversity of class I molecules and increase the repertoire of antigenic peptides that could be presented (Transy et al.,1984 and Lalanne et al.,1985). However, all the detected proteins that are generated by alternative splicing arise from the 3' end of the pre-mRNA and not the 5' portion. Further, in all the reported cases of alternatively spliced class I molecules the result is an amino acid backbone that is either shorter or longer than the wild type protein. Furthermore, the expression of the alternatively spliced molecules is equal to only 10% of that of the wild type protein (Archibald et al.,1986; Lew et al.,1986; McLuskey et al.,1986; Rogers et al.,1986; Krangel et al.,1986; Vogel et al.,1989; Cianetti et al.,1989; Grossberger et al.,1990; Ishitani et al.,1992 and Ellis et al.,1993). Ulker and co-workers (1990) reported that the canonical mRNA encoding the membrane bound form of the class Ib molecule Qa-2 predominated in non-stimulated mouse tissues but the cultured cell lines express enhanced levels of the truncated mRNA. In fact, in some cell lines almost all the expressed Qa-2 was produced by alternative splicing. Alternative splicing was also reported to exist the BoLA system, where cells transformed with the parasite Theileria annulata showed truncated class I molecules missing 16 a.a. from their cytoplasmic domain (Ellis et al.,1993). Therefore, it seems that alternative splicing could be a

mechanism induced by culturing and/or activation of the cells.

The biological function of the reported alternatively spliced class I molecules is yet undetermined and remains a matter of speculation. Due to the fact that most alterative splice events occur at the 3' end of the pre-mRNA it could reflect a certain function for the cytoplasmic domain of the molecule (Ellis et al.,1990). Additionally, structural variation within the intracellular sequences of class I molecules may lead to different pathways of intracellular processing (Zuniga and Hood,1986). A further result of alternative splicing could be the generation of soluble forms of class I molecules. However, the biological significance of such forms is debatable (Krangel et al.,1986). The form of alternative splicing that is most likely to have a functional importance is the deletion of exon 7 which encodes the phosphorylation site (McLuskey et al.,1986; Ellis et al.,1993). The phosphorylation of class I molecules may indicate a role in receptor signalling. The apparent physical interaction of MHC class I molecules with insulin receptors and other hormones (Fehlmann et al.,1985; Phillips et al.,1986 and Edidin,1986), may implicate them in the transduction of hormone receptor-mediated signalling.

The possibility that the heterogeneity observed with the 1D-IEF patterns of the BoLA molecules was a result of alternative splicing, was assessed indirectly. Due to the fact that alternatively spliced molecules show differences in size it was reasoned that their expression could, therefore, be verified using a combination of 1D-IEF and SDS-PAGE in 2D electrophoretic analysis. Reported that alternatively spliced BoLA molecules, isolated from Theileria annulata transformed cells, lack 16 residues from their cytoplasmic domain, a change in size that is equivalent to a molecular mass

change of just over 2,000. Such a change in molecular mass could be resolved by our 2D system. The results obtained from the 2D analysis of neuraminidase treated BoLA class I molecules reflected the presence of a number of charge variants that exhibited identical molecular masses (Fig. 4.12). Some variants revealed the usual doublet formation, others revealed a single heavy band of approximately 44,000 in molecular mass. These results indicated that if alternative splicing does occur in the BoLA system it must result in isoforms of identical sizes and that, unlike alternative splicing reported in species such as humans, mice and sheep, the alternative splicing in the BoLA system is the major contributor to the heterogeneity observed. This is unlikely to be the case. We therefore, conclude that the complexity of the 1D-IEF patterns obtained from non-activated, non-cultured bovine PBL is not a result of alternative splicing of pre-mRNA.

5.3. THE EXPRESSED BoLA MOLECULES ARE PRODUCTS OF SEPARATE LOCI:

From the results discussed in the previous section it is clear that the observed 1D-IEF heterogeneity for BoLA class I molecules is not a product of post-translational modifications and is unlikely to be the product of post-transcriptional modifications.

Charge heterogeneity could also reflect differences in the primary sequences of the molecules observed. The use of peptide mapping in the second dimension as a means for differentiating among molecules with different primary sequences was used to investigate whether this was the case with the observed charge variants of the

BoLA system. The results indicated that each of the charge variants has a unique digestion pattern with endoproteinase Glu-C (Figs. 4.13, 4.14 and 4.15). The molecule v1 obtained from two animals with the w10 serotype (Figs. 4.13 and 4.15) has an identical isoelectric point and digestion pattern to a molecule precipitated from an animal exhibiting w10 in another study reported by Joosten and co-workers (1992). This molecule was precipitated using the polymorphic monoclonal antibody IL-A31 raised against a determinant on a w10 associated molecule. We propose that the molecules v1, reported here, and IL-A31 (Joosten et al.,1992) are identical. Furthermore, molecules v3 and v5 (fig. 4.14) and v3 and v4 (fig. 4.15) could also be identical given the identity of their digestion patterns and pI points and from the fact that the animals exhibiting them share the specificity w17. Further, some molecules immunoprecipitated from the same animal exhibited identical digestion patterns (v3 and v5, fig. 4.14 and v3 and v4, Fig. 4.15). Such similarity could be expected between related molecules from the same species especially when taking into account the fact that even the class I molecules of different species share a great deal of similarity. For example, BL-6 has 73-78% homology to HLA-A,B and C sequences and BL-7 has 75-82% homology to HLA-A,B and C molecules (Ennis et al.,1988).

It was also observed that animals sharing the serological type w10 share a number of different charge variants with different digestion patterns, such patterns were seen with immunoprecipitations from cells of other animals exhibiting the w10 serotype (Figs. 4.13 and 4.15). Similar findings were observed for animals sharing the w17 serotype (Figs. 4.14 and 4.15). These results indicate that each serologically defined BoLA specificity consists of a number of component antigens that differ

amongst each other in their primary sequences. It follows from these finding that the allo-antisera defining each serological specificity do not identify individual antigens as is proposed by Bull and co-workers (1989) and Bernoco and co-workers (1992) (are not monospecific). Furthermore, it was observed that the serotypic patterns remained unchanged between animals sharing it, indicating that the component molecules are encoded by alleles that exist in linkage disequilibrium. The number of different charge variants with different digestion patterns observed in individual animals (figs. 4.13, 4.14 and 4.15) suggests that BoLA class I region encodes at least 3 distinct loci.

5.4. THE EXPRESSION PATTERN OF BoLA CLASS I MOLECULES:

The visual comparison of the BoLA 1D-IEF patterns indicates that animals with similar serological types share very similar banding patterns (Figs. 4.16, 4.17 and 4.18). these patterns could be dissected and the various molecules allocated to different serological specificities. Although the majority of different charge variants could be allocated to serological specificities with which they invariably appear, other molecules appeared to be inconsistent in their associations with the various serological specificities. Such molecules exhibit association with a certain serological specificity in some animals but not in others ("?" in Figs. 4.16, 4.18, 4.19 and 4.20). These results indicate that the BoLA class I molecules show strong linkage disequilibrium, however, this linkage is not absolute. It further indicates the expression of more than one class I locus.

The results from immunoprecipitation experiments using W6/32 and the allo-antisera further supported the above findings and gave further indication to the complexity of the BoLA class I region. The molecules recognised by either MAb or allo-antisera display a number of features that make them likely to be the products of a small number of expressed loci. Molecules '10b' and '11b' (Fig. 4.19) were recognised by either the allo-antiserum or the MAb, not both. The affinity of the different allo-antisera towards these molecules was also different. Allo-antiserum 69 reacted more strongly to the 'b' molecules than it did to those seen by W6/32. Allo-antiserum 102, on the other hand, exhibited a weaker reaction to the basic molecules while reacting strongly with the molecules recognised by W6/32. In addition the bands revealed by AAS 69 and 102 (Fig. 4.19) together could not account for all the IEF bands revealed by W6/32. Immunoprecipitations using AAS 66, 97 and 100 each recognised up to five different charge variants (Fig. 4.20a,b). These, unlike the molecules precipitated with AAS 69 and 102, were all recognised by W6/32. Furthermore, in this case it was the monoclonal W6/32 that recognised molecules with more basic pI points, that were not recognised by any of the AAS (18b and 17b, Fig. 4.20). These discrepancies between W6/32 and the AAS suggest the expression of more than one class I locus.

The 1D-IEF patterns produced by W6/32 and/or the AAS are reproducible among animals with identical serotypes in the population of animals studied. This suggests the presence of linkage disequilibrium among the different class I loci in the herd. The limited breeding pool maintained by artificial insemination also plays a role in limiting the number of different serological specificities present in our population,

which would exaggerate the effect of any linkage disequilibrium.

To extend the panel of AAS and improve the ability to distinguish between the products of different BoLA class I loci it may be advantageous to use reciprocal immunisations between animals from genetically distant breeds (Bos taurus and Bos idicus). It is also advantageous to use the results of 1D-IEF typing in future immunisations to refine the available panel of AAS.

5.5. THE BOVINE CLASS I cDNA SEQUENCES: STRUCTURAL ANALYSIS.

The use of the GCG programme "Isoelectric" revealed that the bovine class I molecules have pI points that, as expected, fall within the pH range 5-7. BL-6 and BL-7 (Ennis et al.,1988) had pI points of 5.24 and 5.27 respectively (Fig. 4.26). The sequences KN104 and w10 (Bensaid et al.,1991) had pI points of 5.71 and 6.11 respectively (Fig. 4.28). The higher pI shown by w10 is consistant with the observed migration of w10 associated molecules on 1D-IEF (Figs. 4.16 and 4.19). pBoLA-1 (Brown et al.,1989) had a pI value of 5.74 (Fig. 4.27). These values are calculated for the amino acid backbones alone. Although, it is accepted that in general the class I MHC molecules encoded by different loci cover, on the whole, different ranges of pI points (Yang et al.,1984), overlap is still observed. The results obtained here confirm this and show that bovine class I molecules encoded by different loci, as is expected with BL-6 and BL-7 (Ennis et al.,1988), could have very similar pI values (5.24 and 5.27, respectively).

The use of the programme "peptidestructure" to predict the secondary structure of the available class I sequences revealed that they were in agreement with the predicted structure of HLA-A2 using the same programme. However,"peptidestructure" relies on the CF and GOR (Chou and Fasman,1978 and Garnier et al.,1978, respectively) algorithms which are only 50-60% accurate (Argos,1989). Nevertheless, the fact that the use of the same programme to predict the secondary structure of the HLA-A2 molecule gave a result resembling that obtained for the BoLA sequences indicated that the class I molecules in human and cattle have an identical overall three-dimensional structure (Figs. 4.29-4.34). It, must be stressed, however, that minor deviations will exist as was observed for the HLA and H-2 three-dimensional structures (Bjorkman et al.,1987; Zhang et al.,1992). It should emphasised that the use of this programme with the current sequence data is inadequate to expose any locus specific differences in the secondary structures of the BoLA molecules.

It has been reported that with the exception of H-2K molecules, the transmembrane regions of alleles of the same locus in the HLA and H-2 system have an equal number of residues (Ennis et al.,1988). The inspection of the published transmembrane region sequences for class I BoLA molecules indicates the presence of alleles of three different loci. BL-6 (Ennis et al.,1988), pBoLA-1 (Brown et al.,1989), w10 (Bensaid et al.,1991) and D19.1 and D19.2 (Ellis et al.,1993) all have 37 residues in their transmembrane regions. BL-7 (Ennis et al.,1988) and D19.3 and D19.4 (Ellis et al.,1993) all have 35 residues. KN104 (Bensaid et al.,1991), on the other hand, has 36 residues in the transmembrane region.

5.6. CONCLUSIONS:

From the results presented in this thesis the following could be concluded. The BoLA class I molecules on non-stimulated bovine PBL obtained from Bos taurus animals are glycosylated at a single N-linked position (Asn 86) and that the sugar moiety is of the complex type with up to three terminal sialic acid residues. It further appears that the class I molecules on such cells are not phosphorylated. Furthermore, both glycosylation and phosphorylation are responsible for the observed charge heterogeneity observed on 1D-IEF. The results also suggest that alternative splicing of pre-mRNA is unlikely to generate the 1D-IEF heterogeneity of BoLA class I molecules immunoprecipitated from non-stimulated, non-cultured bovine PBL.

The use of peptide mapping reveals that the molecules observed using 1D-IEF have differences in their primary sequences. The number of different charge variants with different digestion patterns suggests the presence of at least three class I encoding loci. The peptide maps further reveal the existence of linkage disequilibrium in the BoLA class I region.

The visual comparisons of the 1D-IEF patterns of different animals and the use of the different bovine AAS in the immunoprecipitation of class I molecules further support the existence of linkage disequilibrium and augment the notion of the expression of a number of different class I loci. The tight linkage reported above may explain the inability of the allo-antisera to distinguish between individual antigens in microcytotoxicity assays and is likely to obscure the presence of the less polymorphic molecules.

The results from the computer analysis of the available BoLA cDNA sequences indicate that bovine class I molecules possess pI values similar to those of MHC systems of other species (pH range 5-7). Additionally, the comparison of the secondary structures of the BoLA sequences and that of the HLA-A2 molecule, suggests that the three-dimensional structure of bovine class I molecules is similar to that of molecules from the HLA and H-2 systems. Furthermore, the sequences of the transmembrane regions of the BoLA cDNA sequences further confirm the results from the biochemical studies described above which indicate the presence of at least three class I loci.

The findings of this project have established a number of facts that could be used in future and existing work in the BoLA area. It is clear that each BoLA serological type is in fact composed of a number of different molecules encoded by more than one locus and does not define variants of the same molecule. Therefore, the assignment of serological specificities to the "BoLA-A" locus is premature. Furthermore, it appears that the allelic products of the BoLA loci exhibit strong linkage disequilibrium. This and other information from 1D-IEF typing should be taken into account and used to refine the specificity of the available panel of AAS so that they could truly be "operationally monospecific". Such reagents could then be used with more confidence in studying the associations of BoLA molecules with various diseases and production characteristics for selection purposes.

Further efforts should also be given to the identification of the allelic products of the different BoLA loci both on the DNA and the protein level. The system of peptide mapping used here is reproducible and could be refined further for the detailed

biochemical characterisation of individual BoLA polypeptides. This work is only the first step towards understanding the individual characteristics of these molecules which could be useful in the development of safer and more potent drugs and vaccines.

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APPENDICES

APPENDIX 1: MATERIALS.

A.1.1. ANIMALS:

All animals used in these experiments are British Friesians from the Blyth Bank herd and have been serologically typed.

A.1.2. MOUSE MONOCLONAL ANTIBODIES (MAb):

The MAbs used were W6/32 (Serotec), IL-A88, IL-A30, IL-A60, IL-A73 (kind gift from ILRAD) and B1.1G6 (Kind gift from Dr.B.Mallison). The specificities of all the MAb used are given in the table below.

Antibody	Specificity
W6/32	Anti HLA-A,B and C, monomorphic, conformational, monomorphic in cattle.
IL-A88	Anti BoLA-class I, monomorphic, non-conformational.
IL-A30	Anti bovine IgM.
IL-A60	Anti bovine IgG_1.
IL-A73	Anti bovine IgG_2.
B1.1G6	Anti human β_2m, cross reacts with bovine β_2m.

Note: The negative control MAb 4E (anti-HLA-B) has the same isotype as W6/32 (IgG_{2a}) and was a kind gift from Dr.S.Y.Yang.

A.1.3. ALLO-ANTISERA (AAS):

All the ASS were produced from calf-dam reciprocal immunisations. A list of the Edinburgh AAS is given in table A.1 on page 220.

A.1.4. RECIPES FOR SDS-POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE):

A) Stock Acrylamide:BIS (30%T, 2.67%C):

29.2g Acrylamide (Sigma).

0.8g N'N'-Bis Methylene Acrylamide (Sigma).

Make up to 100ml with DH_2O.

B) 1.5M Tris-Cl, pH 8.8:

45.41g Tris base or Trizma (Sigma).

50ml DH_2O

Adjust pH to 8.8 with HCl (Fisons)

Top up to 250ml with DH_2O.

C) 1M Tris-Cl, pH 6.8:

24.22g Tris base.

50ml DH_2O

Adjust pH to 6.8 with HCl

Top up to 200ml with DH_2O.

D) SDS sample buffer:

2% Sodium Lauryl Sulphate or SDS (0.8g) (Fisons).

20% glycerol (8ml) (Fisons).

0.125M Tris pH 6.8 (5ml of 1M solution or 0.6g Tris base)

0.004% Bromophenolblue (BDH).

5% 2-Mercaptoethanol (2ml) (Sigma).

make up to 40ml with DH_2O

E) Running Buffer:

Tris base (6g).

SDS (1g).

Glycine (28g) (FSA).

make up to 1L with DH_2O.

F) Running gel solutions:

The following recipes are enough for 2 mini SDS gels used with the mini Protein II system (BIORAD):

	12% (ml)	15% (ml)
DH_2O	3.35	2.36
1.5M Tris-Cl (pH 8.8)	2.5	2.5
10% SDS solution	0.1	0.1
Acrylamide:bis	4	4.99
10% APS (Sigma)	0.05	0.05
TEMED (Sigma)	0.005	0.005

The above proportions could be manipulated according to the size of the gel needed.

G) Stacking gel solutions:

The following recipes are enough for 2 mini SDS stack gels used with the mini Protein II system (volumes given in ml):

DH_2O	6.2
1M Tris-Cl (pH 6.8)	1.25
10% SDS solution	0.1
Acrylamide:bis	1
10% APS	0.05
TEMED	0.01

The proportions could be manipulated according to the size of the gel needed.

A.1.5. RECIPES FOR ISOELECTRIC FOCUSING (IEF):

A) Acrylamide:Bis (30%T, 5.33%C):

28.4g Acrylamide.

1.6g N'N'-bis methylene acrylamide.

Make up to 100ml with DH_2O.

B) TX114 Lysis buffer:

0.5ml TX114 (Fluka).

10ml 0.5M Tris-Cl pH 7.4 (50mM).

0.5ml 1M $MgCl_2$(5mM) (Fisons).

Make up to 100ml with DH_2O.

C) TX114 Lysis buffer with inhibitors:

Same as described in (B) with the following additions:

0.4mM EDTA (BDH).

10mM NaF (Sigma).

10mM $Na_4P_2O_7$ (Sigma).

0.4mM $NaVO_3$ (Sigma).

1mM PMSF (Sigma).

10mM Iodoacetamide (Sigma).

D) Phosphate free culture medium:

150mM NaCl (Fisons).

5mM $MgCl_2$ (BDH).

5mM KCl (Fisons).

1.8mM Glucose (Fisons).

2mM Glutamine (Sigma).

10mM Tris-acetate pH 7.4.

make up to 100ml with DH_2O.

C) NET buffer solution:

10ml 0.5M tris-Cl pH 7.4 (50mM).

0.5ml 10% Nonidet P40 or NP40 (Fisons).

10ml 1.5M NaCl (150mM).

0.5ml 1M EDTA (5mM) or (1ml 0.5M).

Make up to 100ml with DH_2O (might need warming).

D) IEF Gel:

8.3ml Acrylamide:Bis.

29.7g Urea (Fisons).

11ml 10% NP40.

9ml DH_2O.

Leave in 37C water bath until urea dissolves.

Add ampholines (Pharmacia LKB):

2.2ml pH range 5-7.

0.55ml pH range 3.5-10.

0.22ml pH range 7-9.

Mix well then add:

100ul Ammonium Persulphate.

50ul TEMED.

For mini rod gels (complimentary to the mini Protein II system) use same solutions as above but only 1/10 of the volume.

E) IEF sample buffer:

28.5g Urea.

1ml Ampholine pH range 3.5-10.

1ml 10% NP40.

2.5ml Beta-mercaptoethanol

Make up to 50ml with DH_2O and store at -20C in 1ml aliqouts.

F) Overlay buffer:

1 part IEF buffer.

3 parts DH_2O.

0.1% Bromophenolblue.

G) Top tank buffer:

100ml 0.5M NaOH (Fisons).

Make up to 1L with DH_2O.

H) Bottom tank buffer:

2.2ml commercial H_3PO_4 (Fisons).

Make up to 1L with DH_2O.

I) Apparatus:

Teflon inserts and Aculon spacers.

Glass plates 19cm * 30.5cm.

21cm * 30.5cm.

Bulldog clips.

A.1.6. 2-DIMENSIONAL GEL ELECTROPHORESIS (2D):

Use 12% SDS-PAGE and IEF recipes as above. The apparatus used is the Mini 2D cell (BIORAD).

A) 2D equilibration buffer:

0.125 Tris base (0.76g).

10% glycerol (10g).

2.3% SDS (2.3g).

Make up to 50ml with DH_2O, adjust pH to 6.8 with HCl then top up to 100 with DH_2O.

A.1.7. 2D PEPTIDE MAPPING:

Use 15% SDS-PAGE and IEF recipes as above. The apparatus used is the Mini 2D cell (BIORAD).

A) V8 Protease from Staphylococcus aureus EC 3.4.21.19 (Bohringer Mannheim) made up to a concentration of 0.25mg/ml in 0.125M Tris-Cl pH 6.8 and frozen at -20C as 200ul aliqouts (62.5ug each, 2ul are used for each run).

B) V8 Protease dilution buffer:

2.5ml SDS-PAGE stacking gel buffer.

0.1ml 10% SDS.

1ml glycerol.

20ul 0.5M EDTA.

30ul Beta-mercaptoethanol.

6.3ml DH_2O.

Bromophenolblue (trace).

C) 1% Agarose solution (BRL).

A.1.8. FACS TEST SOLUTIONS:

A) Fluorescent Immunoconjugate (Nordic Laboratories):

i. For MAbs:

Rabbit anti mouse Ig (1/100).

ii. For sera:

Rabbit anti bovine Ig (1/100).

B) FACS medium (100ml):

i. For PBL:

RPMI (Gibco).

5% Horse serum.

0.2% Sodium Azide (BDH).

A.1.9. GENERAL:

A) HBSS solution:

100g HBSS.

0.4ml Sodium Heparin (5000U/ml) (Leo Laboratories Ltd.)

in 900ml of DH_2O and pH to 7 with sodium carbonate (Gibco) then top up to 1L with DH_2O.

B) Ficoll/Hypaque s.g. 1.069:

67.5g Ficoll (Pharmacia LKB) dissolved in 400ml of hot DH_2O then make up to 750ml and leave to cool.

79.8g Sodium Hypaque (Stirling Research) and dissolve in 120ml of warm DH_2O then make up to 200ml and leave to cool.

Add the Sodium Hypaque to the Ficoll solution gradually, stirring all the time until s.g. 1.069 is achieved.

C) ^{14}Carbon Labelled Low Molecular Weight Markers (Sigma).

D) ^{35}Sulpher Labelled Methionine (Amersham). Usually in 66ul that is diluted to final volume of 200ul with 1mM 2-Mercaptoethanol.

E) $^{32}P_i$ carrier free phosphorus (Amersham).

F) Pansorbin cells (Protein A) (Calbiochem).

G) Gamma-bind G agarose, recombinant form of protein G (BDH). Switched to Gamma-bind G sepharose (same product as above but from Pharmacia LKB).

H) Neuraminidase from Vibrio cholerae EC 3.2.1.18 (Behring), 0.1 unit used for each digestion as prepared by manufacturer.

I) Endoglycosidase F/N-Glycosidase F from Flavobacterium meningosepticum EC 3.2.1.96/EC 3.2.2.18. (Bohringer Mannheim).

J) Deglycosylation buffer:

60mM Sodium Acetate pH 5.5 (0.49g) (BDH).

30mM EDTA (1.12g).

1.2% NP40.

0.2% SDS.

1% 2-Mecaptoethanol.

The buffer is added to the enzyme to make a final volume of 50ul.

K) Potato acid phosphatase EC 3.1.3.2 (Calbiochem).

L) Dephosphorylation buffer:

50mM 2-(N-Morpholino)ethanesulphonic acid (MES) pH 6.2 (BDH).

0.1% NP40.

The buffer is added to the enzyme to make a final volume of 200ul.

M) Commercial Dimethyle Sulphoxide (DMSO) (Fisons).

N) PPO/DMSO:

100g 2,5-Diphenyl-Oxazole or PPO (Sigma).

400ml DMSO.

O) Kodak X-OMAT AR X-ray film.

P) Electrophoresis Power supply:

i. Pharmacia LKB constant power supply ECPS 3000/150.

ii. LKB Bromma 2301 Microdrive 1 power supply.

Q) Centrifuges:

i. IEC Centra-8, Refrigerated and Unrefrigerated.

ii. Sorvall RC-5B Refrigerated Superspeed Centrifuge.

iii. Eppendorf 5415.

iv. Sarstedt MH-2K (refrigerated).

APPENDIX 2: FACS ANALYSIS OF BOVINE AAS.

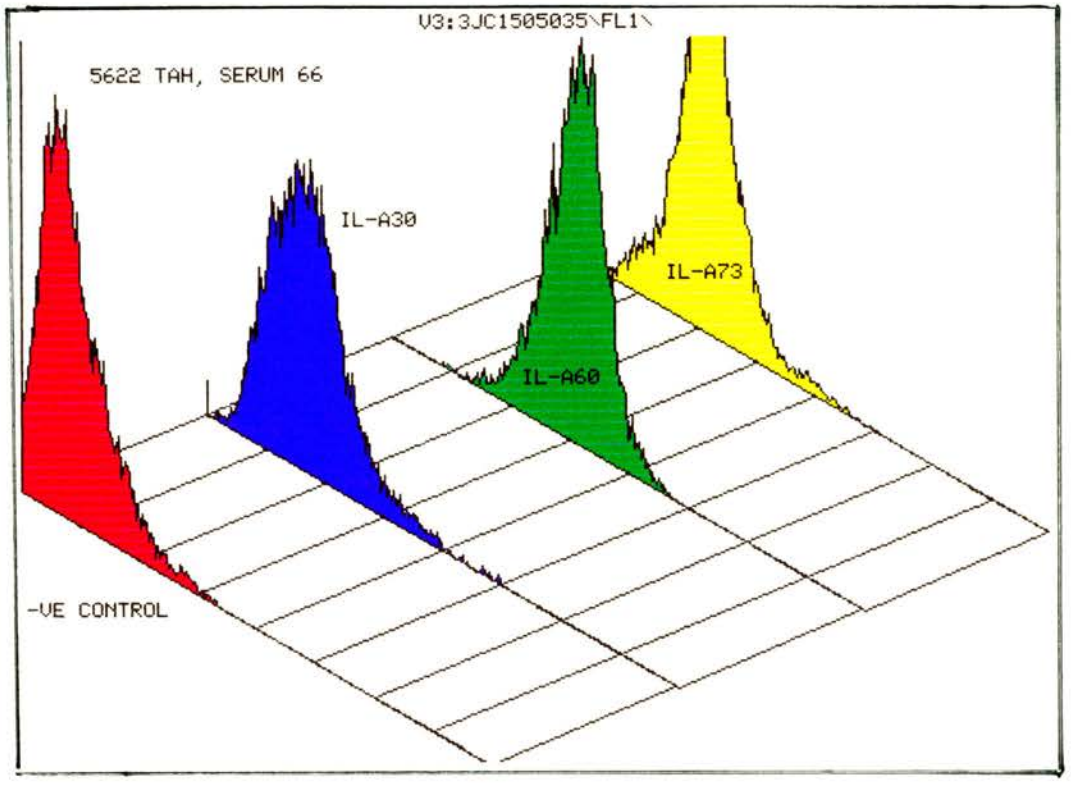
It was observed some bovine AAS were unable to immunoprecipitate class I for 1D-IEF analysis, although they showed positive reactions in lymphomicrocytotoxicity assays. The most likely reason for this observation was that the non-reacting sera had a small proportion of IgG (products of a primary challenge). In order to investigate this possibility, bovine T cell lines transformed by the parasite T. annulata were used to eliminate the possibility of the AAS reacting to surface expressed Ig. The cells were initially incubated the AAS to be tested, followed by a MAb directed against bovine IgM (IL-A30), bovine IgG_1 (IL-A60) or IgG_2 (IL-A73). The final step involves the addition of the fluorescent conjugate. The results are shown in figures A.1 and A.2, and confirmed that the successful sera had higher proportions of IgG than unsuccessful sera. The sera production records further confirmed that the unsuccessful AAS were invariably the products of a primary challenge.

FIGURE A.1
Facing page

ISOTYPE PROPORTION TEST FOR THE SUCCESSFUL IMMUNOPRECIPITATING AAS 66 AND 102.

Figure A.1a, shows the FACS profile for the AAS 66 (w6 broad specificity). Figure A.1b shows the profile obtained with AAS 102 (w11 specific). In both cases the proportions of IgG_1 and IgG_2 are higher than those observed for IgM and the which showed a profile resembling that of the negative control (no AAS).

(A.1a)


V3:3JC1505035\FL1\
5622 TAH, SERUM 66
IL-A30
IL-A73
IL-A60
-VE CONTROL

(A.1b)

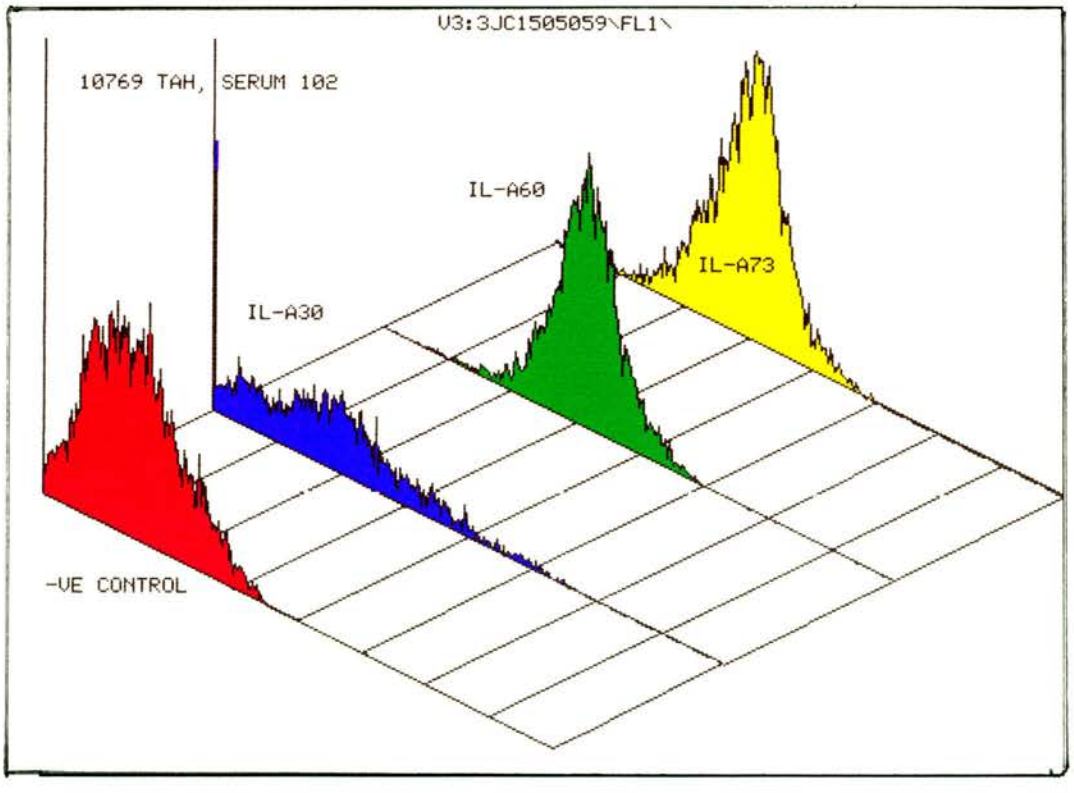
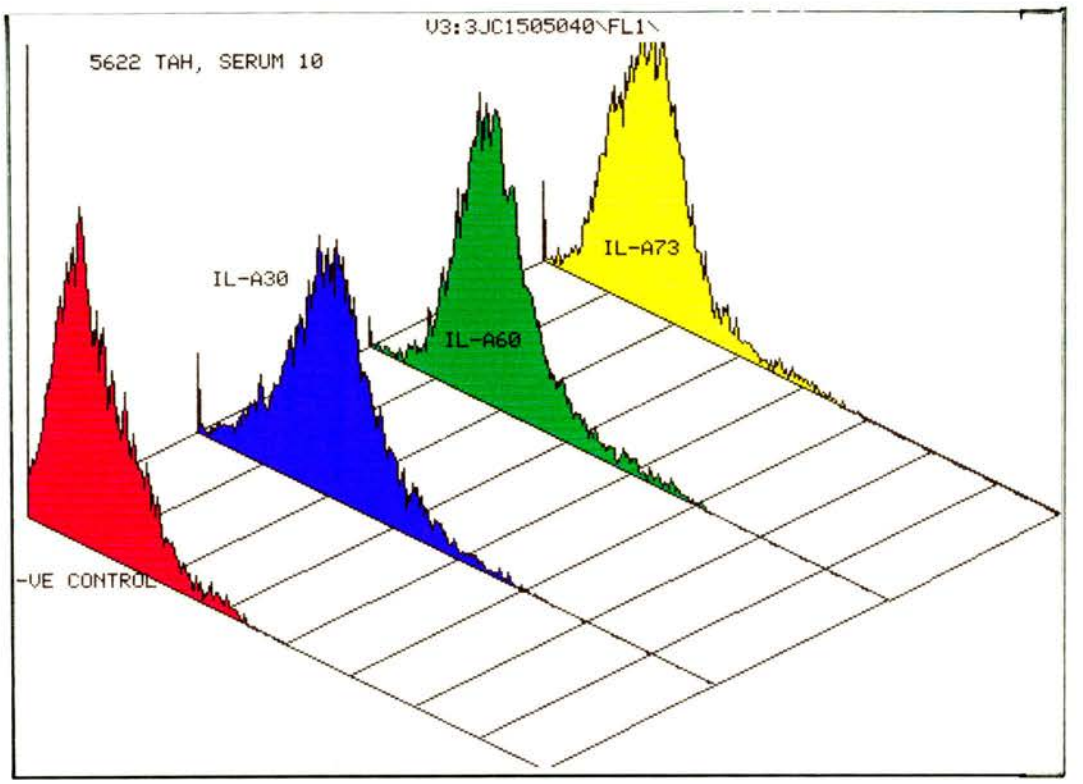

V3:3JC1505059\FL1\
10769 TAH, SERUM 102
IL-A60
IL-A73
IL-A30
-VE CONTROL

FIGURE A.2
Facing page

ISOTYPE PROPORTION TEST FOR THE NON-IMMUNOPRECIPITATING AAS 10 AND 86.

Figure A.2a, shows the FACS profile for the AAS 10 (w6 broad specificity). Figure A.2b shows the profile for AAS 86 (w18 specific). In both cases the relative proportions of IgG_1 and IgG_2 are similar to (A.2a) or less (A.2b) than that of IgM.

(A.2a)


V3:3JC1505040\FL1\
5622 TAH, SERUM 10
IL-A30
IL-A60
IL-A73
-VE CONTROL

(A.2b)

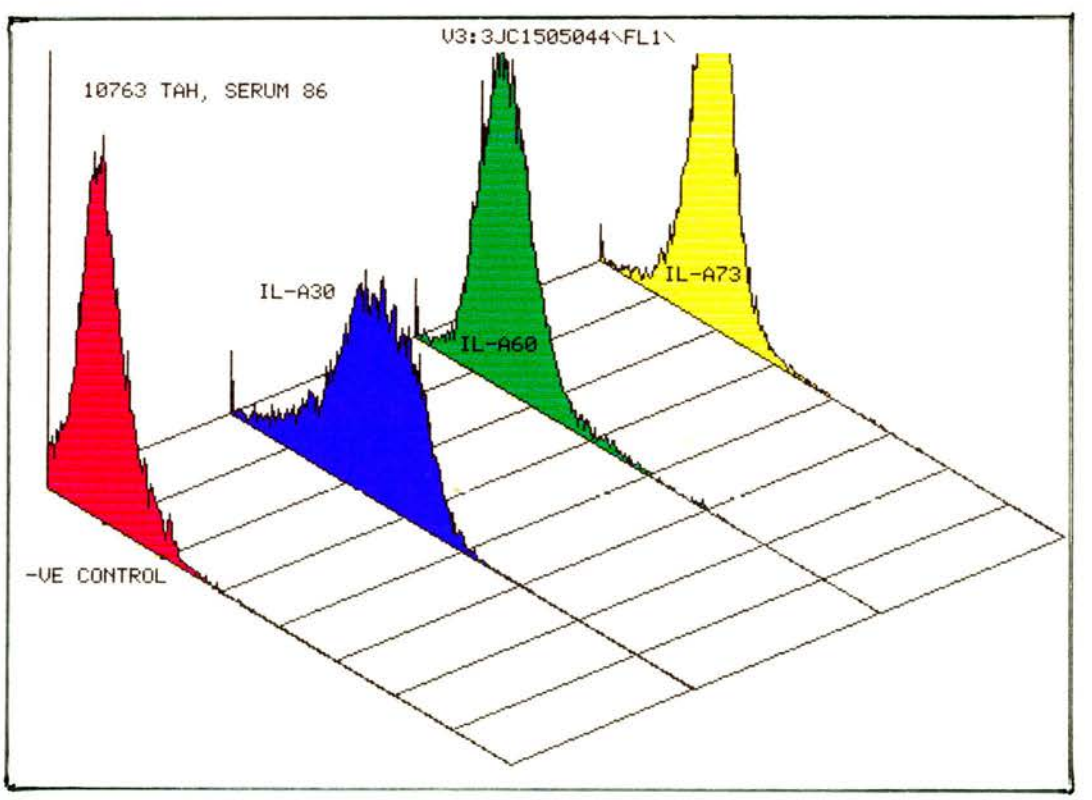

V3:3JC1505044\FL1\
10763 TAH, SERUM 86
IL-A30
IL-A60
IL-A73
-VE CONTROL

Table A.1: **EDINBURGH ALLO-ANTISERA AND THEIR DEFINED SEROLOGICAL SPECIFICITIES.**

SPECIFICITY	DEFINING SERA	ASSOCIATED SERA
w1	1, 8, 83, 151	112
w2	2, 62, 64, 65, 115	
w3	39	
w4	82, 117	
w5	7, 90	
w6	9, 10, 66, 75, 97	
w7	11, 17, 67, 68	
w8	13	
w9	14, 140	
w10	69, 71	
w11	73, 76, 102, 110	
w12	47, 133	
w13	5	
w14	80, 138	16, 136, 101, 109, 136
w15	105, 152	
w16	38, 43, 114, 131	
w17	12, 92, 93	
w18	81, 86, 113	
w19	100	
w20	78, 121	
w21	107, 123	120, 125
w22	63	
w25	111, 132	
w30	74	
w32	40, 124, 126	
Eu27	94	
Unknown	30, 85 (w4, w7 and w10)*, 87 (w3)*, 89, 91, 99, 104, 116, 118, 127, 137	

* Possible specificities defined.